بسم الله الرحمن الرحيم

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مركز أبحاث للجينات والتقنية البيولوجية
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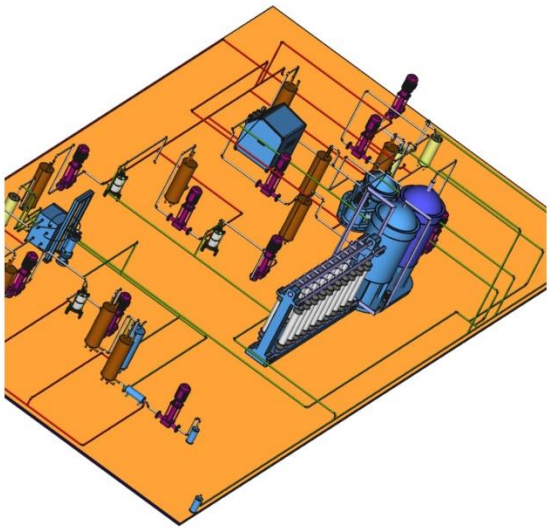
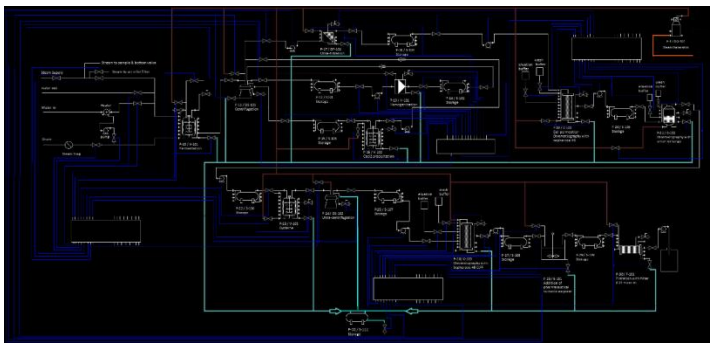
MEGBI-VPP (Vaccine Production Pilot Plant) Final Report (Period 2006 - 2015)

- Development of a synthetic peptid vaccine against H5N1 based on MHC-I epitopes (2006 – 2011)
- Basics in biotechnological upstream an downstream processing, in common DNA vaccine technology, and in Hepatitis B DNA vaccine technology
- Specification, System Design, Detailed Devices and Plant Design, and Automation for the MEGBI Vaccine Pilot Plant MEGBI-VPP (2012 - 2015)



Last Update: 31. August 2020



Based on the following research reports:

[Gafsi 2007] Hadhemi Gafsi, Diplomarbeit „Design eines synthetischen Peptid-Vaccins gegen H5N1 basierend auf MHC-I-Epitopen“,Univ. Bremen/IGEEH, Karlruhe, 2007

[MEGBI-H5N1 2011] Noha Abdulwahab (رحمها الله), Samir Mourad, Samah Borghol, Laboratory verifiying of the peptid candidate FLKDVMESM by ELISA and IFN-γ ELISPOT analysis (Step 2 in MEGBI H5N1 peptid vaccine research project), Ras Nhache, June 2011 (engl./عربي)

[MEGBI-VPP 2012] Samir Mourad, Rihab El Merheb, Layal Chbib, ” *MEGBI Vaccine Pilot Plant – 1st Project Report (Feb 2012 – Jan 2013)”, Introduction to Biotechnological upstream and downstream processing*

مدخل تطبيقي الى البيوتكنولوجيا، **تكثير خلايا في داخلها جينات يراد انتاج بروتينها وتنقية هذه البروتيئينات**، جميع التفاصيل باللغة العربية والمصطلحات العلمية باللغتين العربية والانجليزية

[MEGBI-VPP 2013] Samir Mourad, Jawdat Al Khatib, Rafiq Mourad, Hassan Derbani, *MEGBI Vaccine Pilot Plant – 2nd Project Report (Feb 2013 – Dec 2013),*

[MEGBI-VPP 2014-15] Samir Mourad, *MEGBI Vaccine Pilot Plant – 3rd Project Report*

Content in short

Content

Development of a synthetic peptidvaccine against H5N1 based on MHC-I epitopes

Entwicklung eines synthetischen Peptid-Vaccins gegen H5N1 basierend auf MHC-I-Epitopen
Development of a synthetic peptid vaccine against H5N1 based on MHC-I epitopes
S. Mourad1 , H. Gafsi
Institute for Genetic Engineering, Ecology and Health (IGEEH) /
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The avian influenza virus: H5N1

Avian influenza is caused by the influenza-A-Avirus H5N1 which is found in birds.

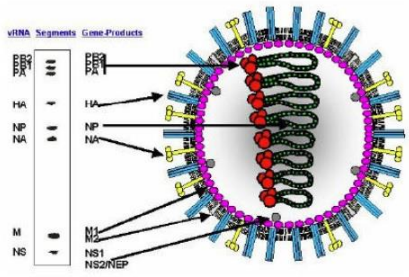

Figure 1: Influenza-A-virion.
The influenza-A-type virus is characterized by the HA (hemaglutinin) and NA (neuraminidase) proteins. H5N1 has one HA type 5 protein (H5) as well as one NA type 1 protein (N1)..

Prerequisites for a potential avian influenza pandemic among humans

There is growing concern that the H5N1 virus might cause a severe pandemic in the near future. Two out of three prerequisites for such a pandemic have already been fulfilled [1]:
● The majority of humans does not possess any kind of immunity against the H5N1 virus and second,
● the virus is able to replicate efficiently inside the human body.

The third prerequesite which has not been fulfilled yet is:
● a fast and effective spreading of H5N1 in the human population through the means of human to human transmission of the virus [2][3].
However, a mutation in the H5N1 virus, leading to these human pathogenic characteristics, could at any time fulfill this third prerequesite [3][4][5].

One of the dreaded potential mutations
Only two amino acid exchanges at the HA receptor binding site of H5N1 are required in order to optimize binding of the virus to N-acetyl-neuraminic acid, which is found on epithelial cells of the human lung [6]. These mutations would enable direct human to human transmission of the H5N1 virus.

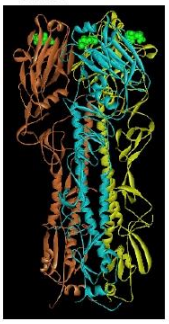

Figure 2. Depiction of the trimeric HA protein
Interaction of HA with NA (in green) is shown [7].

Goal

Development of a vaccine for H5N1
Identification of peptides of the potential H5N1 mutants which might elicit an immune response in humans. Humans could be immunized with these peptides before the outbreak of a pandemic.

Approach

● Computer-based analysis of candidate peptides of the mutated H5N1 which bind to MHC-I with high affinity and hence are immunogenic.
● Verification of these candidates through ELISA and IFN-γ ELISPOT analysis in a laboratory setting.
● Animal testing of the candidate peptides which have found to be promising in the above mentioned tests.

References

[1] http://www.who.int/csr/disease/avian_influenza/phase/en/
[2] Claas, E. C., Osterhaus, A. D., van Beek, R., De Jong, J. C. Rimmelzwaan, G. F., Senne, D. A., Krauss, S., Shortridge, K. F., Webster, R. G. (1998) Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. *Lancet 351(9101):472-477.*
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[4] Harvey, R., Martin, A. C., Zambon, M., Barclay, W. S. (2004) Restrictions to the adaptation of influenza a virus h5 hemagglutinin to the human host. *J Virol., 78: 502-7.*
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[6] Wang, M., Lamberth, K., Harndahl, M., Roder, G., Stryhn, A., Larsen, M. V., Nielsen, M., Lundegaard, C., Tang, S. T, Dziegiel, M. H. (2007) CTL epitopes for influenza A including the H5N1 bird flu; genome-, pathogen-, and HLA-wide screening. *Vaccine, 25(15): 2823-2831.*
[7] Gao, W., Soloff, A. C., Lu, X., Montecalvo, A., Nguyen, D. C., Matsuoka, Y., Robbins, P. D., Swayne, D. E., Donis, R. O., Katz, J. M., Barratt-Boyes, S. M., Gambotto, A. (2006) Protection of Mice and Poultry from Lethal H5N1 Avian Influenza Virus through Adenovirus-Based Immunization. *Journal of Virology 80(4):1959–1964.2006.*
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Methods

Computer-based MHC-I prediction

● Prediction of immunogenic MHC-I epitopes from the modified H5N1 protein by NetCTL 1.2.
● The same method of prediction was also used for the H5N1 nucleoprotein (NP), the polymerase protein (PB1) and the matrix protein (M1), since these proteins also harbor MHC-I epitopes in their amino acid sequence.
● The NetCTL 1.2 prediction has been employed for all pre-defined 12 HLA (human leukocyte antigen) types; these encompass 99% of MHC-I alleles of the entire human population.

Figure 2. The NetCTL 2.1 Server

ELISA Experiment

The actual binding affinity between the synthetical peptides and the MHC-I alleles from the respective HLA types for which peptides have been predicted by NetCTL 1.2, need to be investigated by using quantitative ELISA (see Sylvester-Hvid et al., 2002 [9]).
In order to do this, all candidates (9mer peptides) need to be synthesized with a purity of 95%.

Animal studies and IFN-γ ELISPOT analysis

The immunogenic potency of the peptides needs to be verified using IFN-γ ELISPOT analysis (see Wang et al., 2007). For this purpose we are planning to utilize murine PBMC cells (*peripheral blood mononuclear cells)* infected with the H5N1 virus (see Gao et al., 2006).
To assess the effectiveness of the peptide vaccine, mice need to be treated with the MHC-I peptides which had been verified to be immunogenic. Subsequently these mice will be infected with the H5N1 virus. As a control, mice which have not received the vaccine, will be infected with H5N1.

Clinical Studies
Clinical studies are crucial, since results from animal studies cannot always be directly extrapolated to humans. For example, the majority of substances (about 90%) which had proven safe in animal studies failed in successive human trials. The typical duration of clinical studies is: Phase I – several weeks; Phase II – weeks to months, and Phase III – months up to several years.

Preliminary Results and Discussion

Identification of candidate epitopes using the computer-based MHC-I epitope prediction method

The number of epitopes for the 12 HLA types, which are hypothesized to being presented as CTL ligands in humans, range from n=193 for the modified H5 protein, n=189 for the NP protein, n=272 for the PB1 protein and n=85 for the M1 protein, with a total of n= 739 epitopes.
Of these, n=12 had also been identified as true CTL ligands in a study published by Wang et al., 2007) [7]. These 12 immunogenic epitopes are highly conserved and found in a variety of Influenza A subtypes, so also in the H5N1 virus.
20 epitopes which have been predicted for the modified H5 protein are also found in the sequence stretch of H5 which has been shown to be immunogenic by Gao et al., 2006 [8]. It is therefore highly likely that these are true CTL ligands, able to trigger an immune reaction in humans, and consequently potent to confer protective immunity for H5N1.
Two of these epitopes harbor the mutation found in the modified H5 protein. However, these epitopes only bind to two HLA types, HLA-B27 und -39.
It can be concluded that the method used in this study (using mutated H5 which is adapted to humans in its receptor binding site, and subsquently designing a vaccine from these H5 epitopes for protection from the human-adapted version of H5N1) might not prove successful.

Summary and Discussion

32 peptides wich are true CTL ligands and hence valid candidates for a MHC-I based vaccine, have been identified in this study. Theses peptides bind to 11 out of 12 HLA types, leaving only HLA type B44 with no true immunogenic CTL ligand identified. Nevertheless, the 11 HLA types A1, A2, A3, A24, A26, B7, B8, B27, B39, B58 and B62 cover 99% of HLA alleles of humans of all ethnicities. This means that a peptide vaccine based on these 32 epitopes could most likely confer sufficient immunity against H5N1 in humans.
Due to the highly conserved epitopes found in the NP, PB1 and M1 proteins of H5N1, such a vaccine might also protect from a human-adapted version of H5N1.

Verification of these data by ELISA experiments, animal studies and clinical studies are currently being planned.

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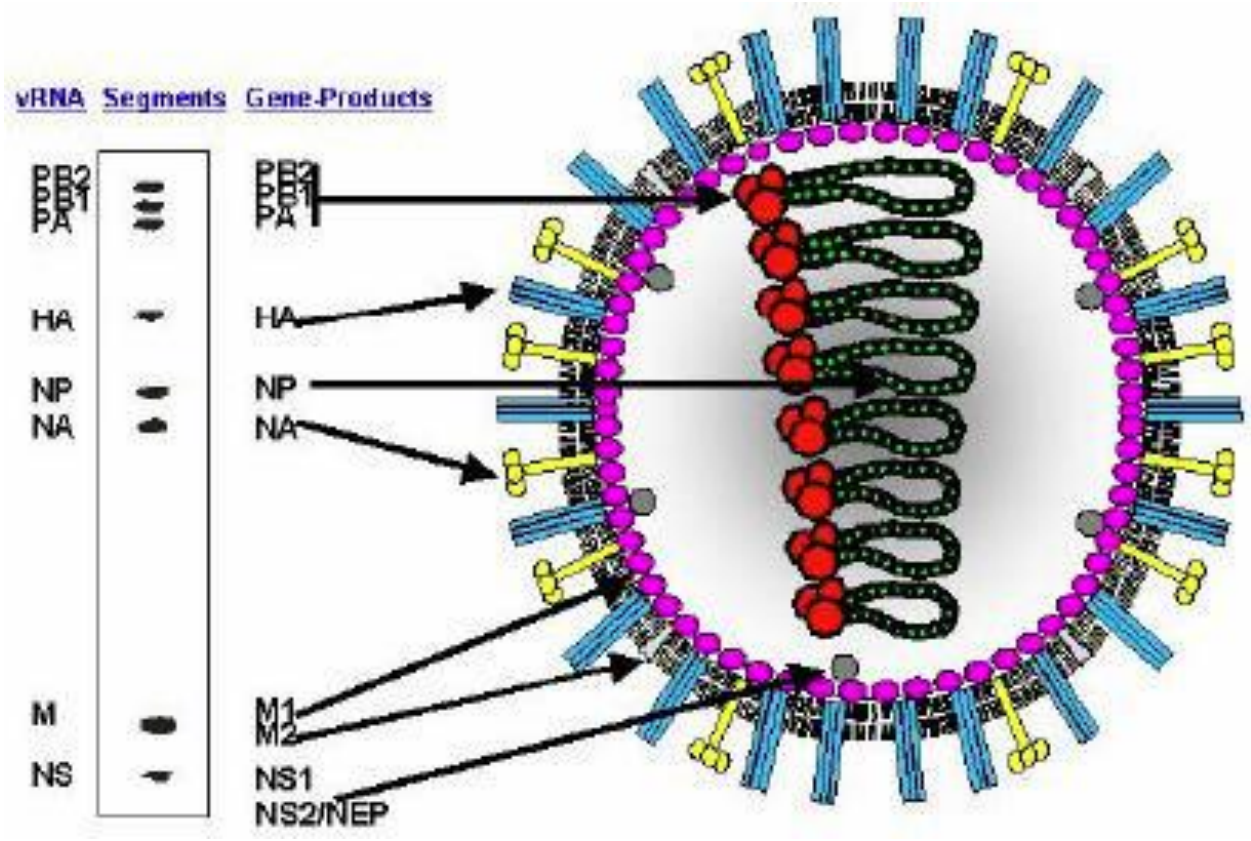

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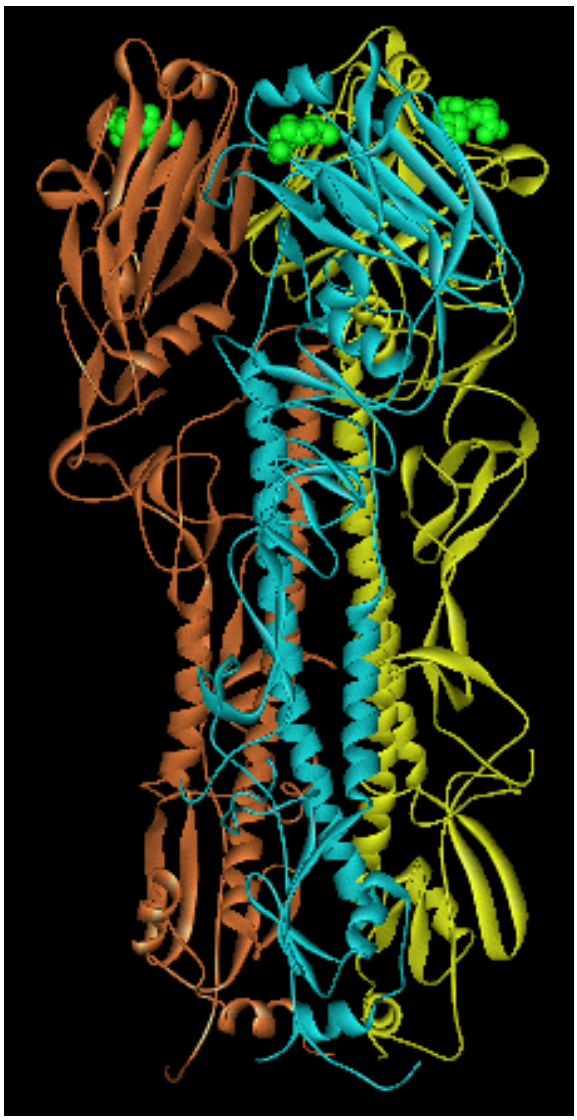

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Mice and Poultry from Lethal H5N1 Avian Influenza Virus through Adenovirus-Based Immunization. *Journal of Virology 80(4):1959–1964.2006.*

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1 Computer-based analysis of candidate peptides of the mutated H5N1 which bind to MHC-I with high affinity and hence are immunogenic

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Diplomarbeit

Design eines synthetischen Peptid-Vaccins gegen H5N1 basierend auf MHC-I-Epitopen

Hadhemi Gafsi

Bremen, 29. April 2007

Betreuer: Dipl.-Ing. Dipl.-Inform. Samir Mourad (VGÖG e.V., Karlsruhe)

1. Gutachter: PD Dr. Andreas Dotzauer
2. Gutachter: Prof. Dr. Angelika Vallbracht

Studiengang: Diplom-Biologie, Fachbereich 2, Universität Bremen

2 Verification of the peptid candidate FLKDVMESM through ELISA and IFN-γ ELISPOT analysis in a laboratory setting

Task description:

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Middle East Genetics and Biotechnology Institute (MEGBI)
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تطوير لقاح الببتيد المصنع ضد فيروس إنفلونزا الطيور المستند على حواتم أليلات المركب الرئيسي للتلاؤم النسيجي.(MHC-I).

إن فيروسات إنفلونزا آي (H5N1) تسبب إنفلونزا الطيور. ويميز هذه الفيروسات النوع الخامس من البروتيينات السكرية الراصة الدموية (H5 hemaglutinin) والنوع الأول من بروتيينات النوروأميديناز (Neuraminidase N1) المغلفة للمادة النووية للفيروس.

وبما إن غالبية البشر لا تملك أي نوع من الحصانة ضد فيروس H5N1 فإن هذا الفيروس قادر على التكرار بكفاءة داخل جسم الإنسان لذا يجب مواجهة هذا الوباء. إلا أن التغيير الجيني المفاجئ في الفيروس من خلال تبادل الأحماض الأمينية في مستقبل موقع ملزم لفيروس h5n1 من أجل تحسين ربط الفيروس بحمض أن-أسيتيل نورامينيك الذي يوجد على الخلايا الظاهرية لرئة الإنسان، يسبب الإنتشار السريع والفعال له بين البشر.

لكيفية تطوير هذا اللقاح يجب معرفة ببتيدات طفرات H5N1 المحتملة التي قد تثير رد فعل مناعة لدى البشر.

المنهج:

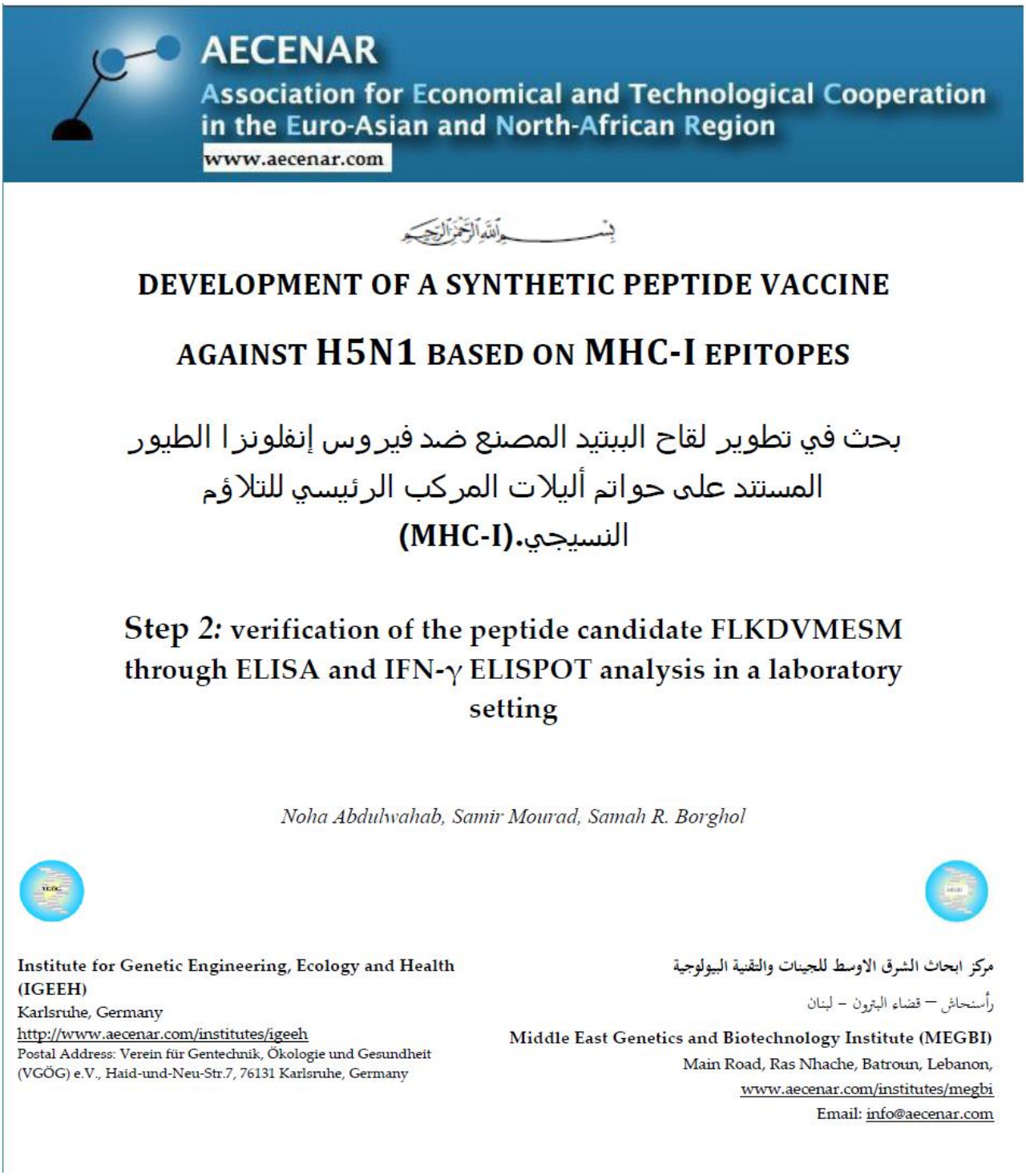
- يستند الحاسوب على تحليل الببتيد المرشح للتغير الجيني المفاجئ في فيروس H5N1 الذي يرتبط ب MHC-I في نسبة عالية لذا فهي المناعة.
- التحقق من هذه المرشاحات من خلال فحص إيليزا و IFN-Y ELISPOT في المختبر.
- إختبار الببتيدات المرشحة، التي وجدت في الفحوصات المذكورة أعلاه لتكون محفزة للمناعة، على الحيوان.

الطرق

المرحلة الأولى: هي إستناد الحاسوب على تنبؤ MHC-I تم إنجاز ها في ألمانيا.
المرحلة الثانية: هي إختبار إيليزا الكمي للتحقق من نسبة الربط الفعلي بين الببتيدات المصنعة وأليلات المركب الرئيسي للتلاؤم النسيجي (MHC-I) من مستضدات كريات البيض البشرية (HLA) للببتيدات التي تم تنبؤها بواسطة NetCTL 1.2.
المرحلة الثالثة: هي الدراسات الحيوانية وتحليل IFN-Y ELISPOT للتحقق من فعالية لقاح الببتيد.
المرحلة الرابعة: هي الدراسات السريرية

Master Thesis

المرحلة الثانية وقسم من المرحلة الثالثة هما الآن قيد البحث وقد يستغرقان مدة ستة أشهر أو أكثر لإنجاز هما وسيتم العمل عليهما إن شاء الله في مختبر MEGBI في رأسنحاش-البترون ولمزيد من الإستفسار الإتصال على: 06/921318 أو الإتطلاع على
http://aecenar.com/download/doc_download/45-h5n1-peptide-vaccine-research-research-plan-step-2-pdf



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بسم الله الرحمن الرحيم

DEVELOPMENT OF A SYNTHETIC PEPTIDE VACCINE AGAINST H5N1 BASED ON MHC-I EPITOPES

بحث في تطوير لقاح الببتيد المصنع ضد فيروس إنفلونزا الطيور المستند على حواتم أليلات المركب الرئيسي للتلاؤم النسيجي.(MHC-I)

Step 2: verification of the peptide candidate FLKDVMESM through ELISA and IFN-γ ELISPOT analysis in a laboratory setting

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Last update: 2 June 2011

2.1 Overview / *نظرة عامة*

إن بحث تطوير لقاح الببتيد المصنع ضد فيروس إنفلونزا الطيور المستند على حواتم أليلات المركب الرئيسي للتلاؤم النسيجي.(MHC-I)، هو بحث يستوجب أربع مراحل لإتمامه منها المرحلة الثانية المشروحة بالتفصيل في هذا الكتاب والتي هي إختبار إيليزا الكمي للتحقق من نسبة الربط الفعلي بين الببتيدات المصنعة وأليلات المركب الرئيسي للتلاؤم النسيجي (MHC-I) من مستضدات كريات البيض البشرية (HLA) للببتيدات التي تم تنبؤها في المرحلة الأولى بواسطة NetCTL 1.2.

يتضمن هذا الكتاب في القسم الأول منه المفاهيم والمصطلحات العلمية المستخدمة في البحث، القسم الثاني يتضمن البروتوكولات المستخدمة لتطبيق هذه المرحلة وهي مأخوذة من مواقع عن الإنترنت أما القسم الثالث يتضمن نفس بروتوكولات القسم الثاني مع إجراء بعض التعديلات عليها بما يتناسب مع الآلات والمواد المؤسس عليها المختبر أما القسم الرابع يعرض النتائج الأخيرة مع الصور من التجارب التي تم العمل عليها في مختبر MEGBI وأخيراً مضاف إلى هذا الكتاب الجدول الزمني المستغرق لإتمام كل عملية من هذه المرحلة.

والله ولي التوفيق.

2.2 Introduction / *المقدمة*

Avian influenza is caused by the influenza-A-A virus H5N1 which is found in birds. The influenza-A-type virus is characterized by the HA (hemagluttinin) and NA (neuraminidase) proteins. H5N1 has one HA type 5 proteins (H5) as well as one NA type 1 protein (N1).

The majority of humans do not possess any kind of immunity against the H5N1 virus. The virus is able to replicate efficiently inside the human body, for this reason should be opposite this pandemic. However, a mutation in the H5N1 virus, only two amino acid exchanges at the HA receptor binding site of H5N1 are required in order to optimize binding of the virus to N-acetyl-neuraminicacid, which is found on epithelial cells of the human lung. It causes a fast and effective spreading of H5N1 in the human population.

And the **goal of the research** is the identification of peptides of the potential H5N1 mutants which might elicit an immune response in humans. Humans could be immunized with these peptides before the outbreak of a pandemic.

Approach

●Computer-based analysis of candidate peptides of the mutated H5N1 which bind to MHC-I with high affinity and hence are immunogenic.

●Verification of these candidates through ELISA and IFN-γ ELISPOT analysis in a laboratory setting.

●Animal testing of the candidate peptides which have found to be promising in the above mentioned tests.

إن فيروسات إنفلونزا آي (H5N1) تسبب إنفلونزا الطيور. ويميز هذه الفيروسات النوع الخامس من البروتيينات السكرية الراصة الدموية (H5 hemaglutinin) والنوع الأول من بروتيين النوروامينيداز (Neuraminidase N1) المغلفة للمادة النووية للفيروس.

وبما إن غالبية البشر لا تملك أي نوع من الحصانة ضد فيروس H5N1 فإن هذا الفيروس قادر على التكرار بكفاءة داخل جسم الإنسان لذا يجب مواجهة هذا الوباء. إلا أن التغيير الجيني المفاجئ في الفيروس من خلال تبادل الأحماض الأمينية في مستقبل موقع ملزم لفيروس h5n1 من أجل تحسين ربط الفيروس بحمض أن–أسيتيل نورامينيك الذي يوجد على الخلايا الظاهرية لرئة الإنسان، يسبب الإنتشار السريع والفعال له بين البشر.

لتطوير هذا اللقاح يجب معرفة ببتيدات طفرات H5N1 المحتملة التي قد تثير رد فعل مناعة لدى البشر.

المنهج:

- يستند الحاسوب على تحليل الببتيد المرشح للتغير الجيني المفاجئ في فيروس H5N1 الذي يرتبط ب MHC-I في نسبة عالية لذا فهي المناعة.
- التحقق من هذه المرشاحات من خلال فحص إيليزا و IFN-Y ELISPOT في المختبر.
- إختبار الببتيدات المرشحة، التي وجدت في الفحوصات المذكورة أعلاه لتكون محفزة للمناعة، على الحيوان.

المراحل

المرحلة الأولى: هي إستناد الحاسوب على تنبؤ MHC-I تم

Steps

Step 1 Computer-based MHC-I prediction (worked in Germany).
Step 2 quantitative ELISA Experiment for investigated from the actual binding affinity between the synthetically peptides and the MHC-I alleles from the respective HLA types for which peptides have been predicted by NetCTL1.2.
Step 3 Animal studies and IFN-γ ELISPOT analysis.
Step 4 Clinical Studies

The second step is the step described here and it is now under discussion

إنجازها في ألمانيا.

المرحلة الثانية: هي إختبار إيليزا الكمي للتحقق من نسبة الربط الفعلي بين الببتيدات المصنعة وأليلات المركب الرئيسي للتلاؤم النسيجي (MHC-I) من مستضدات كريات البيض البشرية (HLA) للببتيدات التي تم تنبؤها بواسطة NetCTL 1.2.

المرحلة الثالثة: هي الدراسات الحيوانية وتحليل IFN-Y ELISPOT للتحقق من فعالية لقاح الببتيد.

المرحلة الرابعة: هي الدراسات السريرية

المرحلة الثانية هي المرحلة الوارد ذكرها في هذا الكتاب وهي الآن قيد البحث

2.3 Theoretical basis / *النظرية/الاساسية*

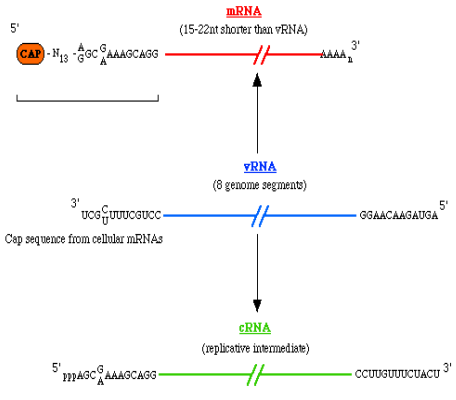
2.3.1 Influenza-A-virus H5N1 / انفلونزا "آي" للفيروس H5N1

The avian influenza is causes by the influenza–A-virus H5N1 that belongs to the family of Orthomyxoviruses

الانفلونزا "آي" H5N1 هي التي تسبب انفلونزا الطيور الذي ينتمي إلى عائلة Orthomyxoviruses

2.3.2 Structure of virus particles and the genome of influenza A virus / هيكل جزيئات الفيروس وجينوم فيروس الانفلونزا آي

- The influenza virus consists of 8 negative-strand RNA molecules surrounded by an envelope. The envelope contains the HA and NA proteins.
- Influenza enters cells by receptor-mediated endocytosis
- Once inside the host, the viral RNAs are [2]



- فيروس الانفلونزا يتكون من 8 سلالات الحمض النووي الريبي السلبية محاطة بمغلف. المغلف يحتوي على البروتينات ها HA و نا NA.
- الأنفلونزا يدخل الخلايا بوساطة.مستقبلات الالتقام.

[2] *www.microbiologybytes.com/virology/Or*

transcribed in the nucleus, stealing 5' caps from host mRNAs. These are then translated at ribosomes.

- On the outside surface of the influenza virus is a lipid bilayer with HA, NA and M2 proteins inserted into it. Inside the bilayer are eight separate, linear RNA segments that make up the viral genome[1].

- ينسخ الحمض النووي الريبي الفيروسي في النواة، مرة واحدة داخل الخلية المضيفة ويأخذ قبعة 5' من الحمض النووي الريبي المضيف. ثم يتم تحويلها الى ريبوسومات

السطح الخارجي لفيروس الانفلونزا هو طبقة ثنائية من الدهون مع HA و NA و البروتينات M2 المدرجة فيه. داخل هذه الطبقة الثنائية شرائح الحمض النووي الريبي الثمانية التي تتشكل الجينوم الفيروسي.

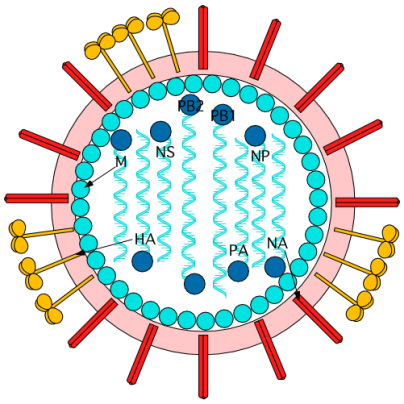
[1] www.microbiologytext.com/index

2.3.3 Genome structure of influenza virus / هيكل الجينوم لفيروس الانفلونزا

Segment:	Size(nt)	Polypeptide(s)	Function
1	2341	PB2	Transcriptase: cap binding
2	2341	PB1	Transcriptase: elongation
3	2233	PA	Transcriptase: protease activity (?)
4	1778	HA	Haemagglutinin
5	1565	NP	Nucleoprotein: RNA binding; part of transcriptase complex; nuclear/cytoplasmic transport of vRNA
6	1413	NA	Neuraminidase: release of virus
7	1027	M1	Matrix protein: major component of virion
		M2	Integral membrane protein - ion channel
8	890	NS1	Non-structural: nucleus; effects on cellular RNA transport, splicing, translation. Anti-interferon protein.
		NS2	Non-structural: nucleus+cytoplasm, function unknown

[Genetics of influenza viruses](). Ann Rev Genet. 2002 36: 305-332.

A representation of the flu virus that shows the outer shell and a cutaway revealing the 8 RNA pieces that comprise the genome of the virus. The outer shell is composed of lipids obtained from the last host cell.



هذا رسم لفيروس الانفلونزا الذي يظهر الغلاف الخارجي ويكشف عن 8 قطع من الحمض النووي الريبي التي يشكل الجينوم للفيروس. وتتألف القشرة الخارجية من الدهون التي تم الحصول عليها من الخلية المضيفة الماضية.

This is decorated with hemagglutinin (HA, yellow) and neuraminidase (NA, pink). HA is necessary for entrance into cells, while NA is needed for release from cells. NP is the viral polymerase. These copies the RNA genome into mRNA for protein synthesis

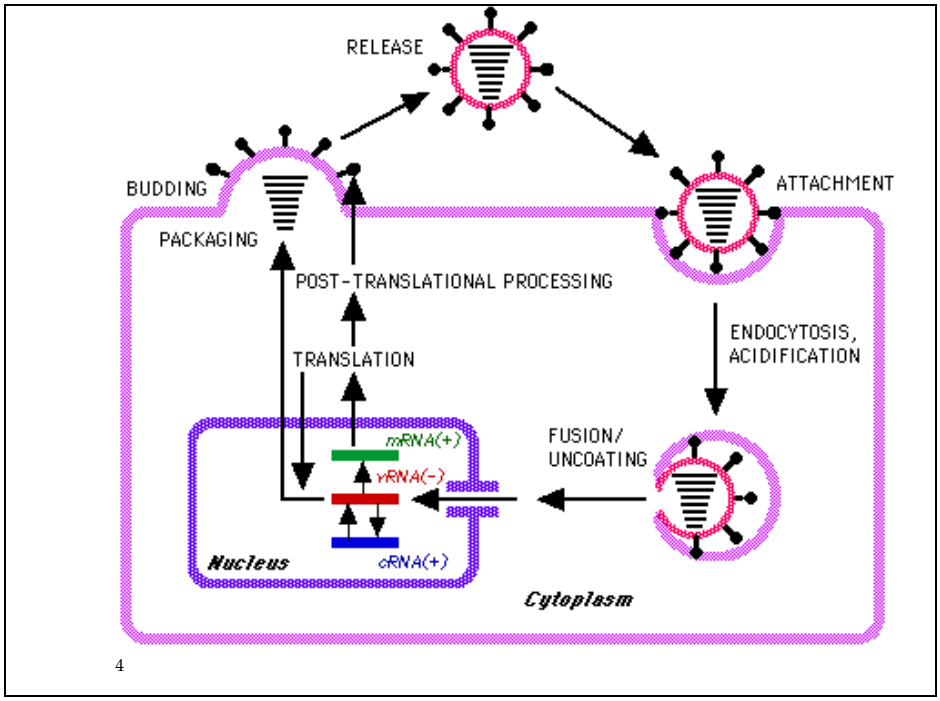
ويزين هذا مع هيماغلوتينين (ها، أصفر) والنورامينيداز (نا، وردي). ها هو ضروري للدخول إلى الخلايا ، في حين أن NA يساعد على الخروج منها. النيكليوبروتين (NP) هو بوليميراز الفيروس. هذه النسخ من الرنا الجينومي داخل الرنا "م" لتصنيع البروتين وبعد ذلك في دورة

and later in the life cycle makes copies of each RNA for new viral particles. NP is very error prone and creates many mutations in the viral genome. HA and NA therefore change rapidly, and escape recognition by our immune systems.

The genome of the virus is composed of eight (-) single-stranded RNAs (these (-) strands cannot be translated into protein) with each segment being complementary to one mRNA. Six of the eight mRNAs code for single proteins, while the remaining two code for two proteins by differential splicing of the RNA. Each mRNA segment is associated with multiple copies of the nucleocapsid protein (NP) and an RNA polymerase (made from the viral proteins PB1, PB2 and PA)[3].

الحياة يجعل نسخ لكل حمض نووي ريبي لجزيئات فيروسية جديدة. نيكليوبروتين هو معرض جدا للخطأ ويخلق العديد من الطفرات في الجينوم الفيروسي وبالتالي الطفرات. "ها" و "نا" تتغير بسرعة، لتهرب قبل أن تتعرف عليها أنظمة المناعة لدينا.

ويتكون الجينوم للفيروس من ثمانية سلالات سلبية للرنا المفرد (هذه السلالات السلبية لا يمكن أن تترجم إلى فروع البروتين) مع كل قطعة يتم اكتمال mRNA واحد. ستة من ثمانية mRNA ترمز لنوع واحد من البروتينات، في حين أن الإثنان المتبقيان يرمزان إلى إثنين من البروتينات بواسطة الربط التفضلي من الحمض النووي الريبي. ويرتبط كل جزء من mRNA مع نسخ متعددة من النيكلويوبروتين وبوليميراز الحمض النووي الريبي (مصنوعة من بروتينات فيروسية PB1، PB2 وPA).



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[3] www.microbiologytext.com/index

[4] Source: www.microbiologytext.com/index

1. Adsorption

The virus becomes attached to the cells, and at this stage, it can be recovered in the infectious form without cell lysis by procedures that either destroy the receptors or weaken their bonds to the virions. Animal viruses have specialized attachment sites distributed over the surface of the virion e.g. orthomyxoviruses and paramyxoviruses attach through glycoprotein spikes, and adenoviruses attach through the penton fibers. Adsorption occurs to specific cellular receptors. Some receptors are glycoproteins, others are phospholipids or glycolipids. These are usually macromolecules with specific physiological functions, such as complement receptors for EBV. Whether or not receptors for a certain virus are present on a cell depends on the species, the tissue and its physiological state. Cells lacking specific receptors are resistant. Attachment is blocked by antibodies that bind to the viral or cellular sites involved.

2. Penetration

Penetration rapidly follows adsorption, and the virus can no longer be recovered from the intact cell.

The most common mechanism is receptor mediated endocytosis, the process by which many hormones and toxins enter cells

The virion is endocytosed and contained within a cytoplasmic vacuole

3. Uncoating

A key step in uncoating is the acidification of the content of the endosome to a pH of about 5, owing to the activity of a proton pump present in the membrane

The low pH causes rearrangement of coat components, which then expose normally

1.الالتصاق

في هذه المرحلة تلتصق الفيروس على الخلية ويمكنها إستعادة عافيتها على الشكل المعدي دون تحلل الخلية بواسطة الاجراءات التي تدمر المستقبلات أو تضعف روابطهم الى الاجسام الفيروسية. الفيروسات الحيوانية تمتلك مواقع متخصصة للالتصاق موزعة على سطح الجسم الفيروسي.مثل paramyxoviruses و orthomyxoviruses يلتصقان من خلال الجليكوبروتين، و adenoviruses بلتصق من خلال الالياف الخماسية. الالتصاق يحدث لمستقبلات خلايا معينة، بعض المستقبلات هي الجليكوبروتين و الاخرين هم الفوسفوليبيد او الجليكوليبيد. وعادة تكون جزيئات ضخمة ضمن وظائف فيزيولوجية محددة مثل تكملة لمستقبلات EBV. سواء كانت أم لم تكن مستقبلات لفيروسات معينة موجودة على خلية تعتمد على الأنواع ، والأنسجة وحالته الفيزيولوجية. تفتقر الخلايا الى مستقبلات خاصة مقاومة. تم منع التعلق عن طريق المضادات الحيوية التي تربط المواقع المشتركة بين الفيروس او الخلايا .

2.الدخول

الدخول السريع يلي عملية الامتصاص، وهنا لا يستطيع الفيروس من استرداد عافيته من خلايا سليمة.

الالتقام بواسطة المستقبلات هو الالية الاكثر شيوعياً. حيث من خلالها يدخل العديد من الهرمونات و السموم إلى الخلايا .

تُلتقم أجزاء الفيروس داخل تجويف سيتوبلاسمي في الخلية

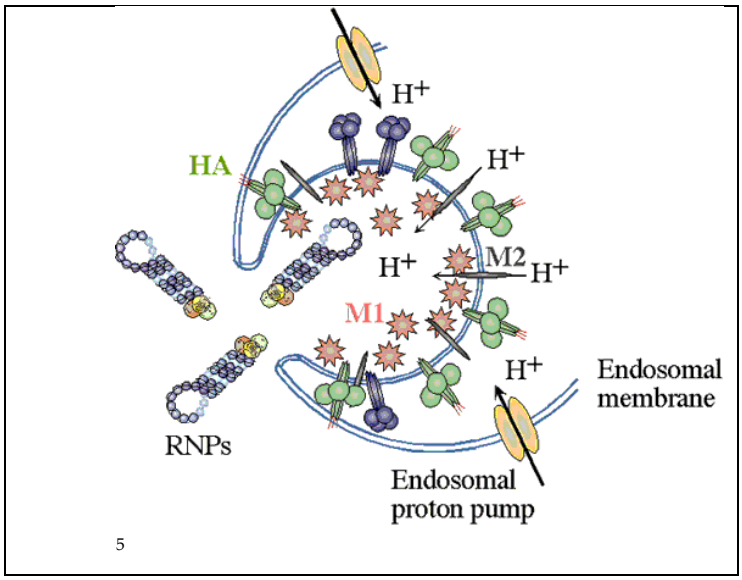
3.نزع الغلاف

الخطوة الاساسية في نزع الغلاف هي في نسبة الأسيد الموجود(PH 5) في الدخلول (هو حيز محاط بالغشاء الخلوي endosome) وذلك بسبب مضخة البروتين الموجودة في الغلاف.

hidden hydrophobic sites.

They bind to the lipid bilayer of the membrane, causing the extrusion of the viral core into the cytosol. For influenza virus, the acid-sensitive component is the core HA_2 unit of the haemagglutinin, for adenoviruses, it is the penton base.

يسبب انخفاض الرقم الهيدروجيني إلى إعادة مكونات الطبقة,الذي يكشف عادة المواقع الغير المائية التي تربط المادة الدهنية في الطبقة الثنائية للغشاء مما تسبب في قدف النواة الفيروسي داخل السيتوسول. ، عنصر الأحماض الحساسة هي وحدة الHA_2 الأساسية من الهيماغلوتينين لفيروس الانفلونزا ، وهي القاعدة الخماسية للadenoviruses



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4. Viral Nucleic Acid Replication

النسخ المتماثل للحمض النووي الفيروسي

Virulent viruses, either DNA and RNA, shut off cellular protein synthesis and disaggregate cellular polyribosomes, favouring a shift to viral synthesis

The mechanism of protein synthesis shut-off varies even within the same viral family

Poliovirus, using a viral protease, causes cleavage of a 200 Kd cap-binding protein, which is required for initiation of translation of capped cellular messengers.

الفيروسات الفتاكة سواء الرنا أو الدنا توقف تصنيع بروتينات الخلية و تفصل البوليريبوسوم لصالح حدوث تحول في تصنيع الفيروس.

إن إيقاف آلية تصنيع البروتين تختلف حتى داخل الأسرة الفيروسية نفسها

فيروس شلل الأطفال يستخدم انزيم لقطع البروتين الفيروسي ويسبب الانقسام لـ200 Kd لقبعة–الملزمة للبروتينات وهو مطلوب للبدء في ترجمة رسائل الخلايا المغطاة.

[5] © Paul Digard, Dept Pathology, University of Cambridge

In contrast to virulent viruses, moderate viruses e.g. polyomaviruses may stimulate the synthesis of host DNA, mRNA, and protein

This phenomenon is of considerable interest for viral carcinogenesis.

وعلى العكس من الفيروسات الفتاكة هناك فيروسات معدلة مثل polyomaviruse يمكنها أن تحفز تصنيع الرنا"م" والدنا و البروتينات للخلية المضافة

هذه الظاهرة أخذت قدرا كبيرا من الإهتمام في السرطان الفيروسي.

Maturation and Release

النضوج و التحرر

Maturation proceeds differently for naked, enveloped, and complex viruses[6]

نضوج الحصيلة بطريقة مختلفة عن الفيروسات المجردة، المغلفة، و المعقدة

2.3.4 MHC-I Molecule / مقعد التوافق النسيجي الكبير الجزئي

- MHC molecules are membrane-bound proteins: MHC I molecules are found on almost all tissues of the body, while MHC II molecules are found only on antigen-presenting cells.
- MHC molecules possess a deep groove that is capable of holding a short peptide. MHC I molecules process proteins present inside the cell and present them on their surface. MHC II molecules present antigens taken from the phagosome digestion, most often foreign cells, and present them to the immune system.

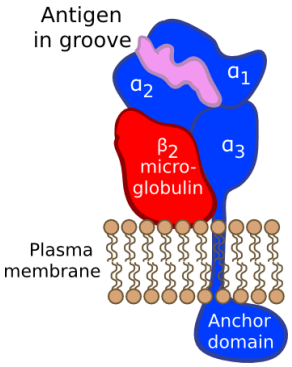
- جزيئات معقد التوافق النسيجي الكبير هي بروتينات غشاء محددة: تم العثور على جزيئات معقد التوافق النسيجي الكبير الأول في أنسجة كلها تقريبا من الجسم، في حين تم العثور على جزيئات معقد التوافق النسيجي الكبير الثاني فقط على مستضد تقديم الخلايا.
- جزيئات معقد التوافق النسيجي الكبير تمتلك تجويف عميق قادر على عقد الببتيد القصير. عملية جزيئات معقد التوافق النسيجي الكبير الأول معالجة البروتينات الموجودة داخل الخلية و عرضها على سطحها. أما جزيئات معقد التوافق النسيجي الكبير الثاني يظهر المستضدات المأخوذة من خلايا الهضم، وفي أغلب الأحيان الخلايا الأجنبية ، وعرضها على الجهاز المناعي.

- The immune system monitors the proteins present on MHC I molecules and activates when a foreign protein, from an intracellular parasite, is detected. This normally results in the destruction of the cell.

- الجهاز المناعي يراقب البروتينات الموجودة على جزيئات معقد التوافق النسيجي الكبير الأول وينشط عندما يتم الكشف عن البروتينات الأجنبية، من الطفيليات داخل الخلايا. هذه النتائج تظهر عادة في تدمير الخلية

Structure of an MHC molecule

[6] http://virology-online.com/general/Replication.htm



2.3.5 β2m./ بيتا 2 ميكروغلوبيلين

This gene encodes a serum protein found in association with the major histocompatibility complex (MHC) class I heavy chain on the surface of nearly all nucleated cells. The protein has a predominantly beta-pleated sheet structure that can form amyloid fibrils in some pathological conditions. A mutation in this gene has been shown to result in hypercatabolic hypoproteinemia[7]

يرمز هذا الجين على بروتين المصل الموجود بالتعاون مع معقد التوافق النسيجي الكبير الصنف الأول للسلسلة الثقيلة على سطح ما يقارب جميع الخلايا التي تمتلك نواة. البروتين له هيكل ورقة مطوية-بيتا في الغالب التي يمكن أن تشكل ألياف نسيجية أميلودية في بعض الحالات المرضية. وقد تبين طفرة في هذا الجين إلى نقص بروتين الدم المفرط الأيض (hypercatabolism)

2.4 Material and Methods

A- Part I

The sequence of HLA-A*0201 was extracted from ncbi: http://www.ncbi.nlm.nih.gov/

```
ORIGIN
        1 atggccgtca tggcgccccg aaccctcgtc ctgctactct cgggggctct ggccctgacc
       61 cagacctggg cgggctctca ctccatgagg tatttcttca catccgtgtc ccggcccggc
      121 cgcggggagc cccgcttcat cgcagtgggc tacgtggacg acacgcagtt cgtgcggttc
      181 gacagcgacg ccgcgagcca gaggatggag ccgcgggcgc cgtggataga gcaggagggt
      241 ccggagtatt gggacgggga gacacggaaa gtgaaggccc actcacagac tcaccgagtg
      301 gacctgggga ccctgcgcgg ctactacaac cagagcgagg ccggttctca caccgtccag
      361 aggatgtatg gctgcgacgt ggggtcggac tggcgcttcc tccgcgggta ccaccagtac
      421 gcctacgacg gcaaggatta catcgccctg aaagaggacc tgcgctcttg gaccgcggcg
      481 gacatggcag ctcagaccac caagcacaag tgggaggcgg cccatgtggc ggagcagttg
      541 agagcctacc tggagggcac gtgcgtggag tggctccgca gatacctgga gaacgggaag
      601 gagacgctgc agcgcacgga cgcccccaaa acgcatatga ctcaccacgc tgtctctgac
      661 catgaagcca ccctgaggtg ctgggccctg agcttctacc ctgcggagat cacactgacc
      721 tggcagcggg atggggagga ccagacccag gacacggagc tcgtggagac caggcctgca
```

[7] http://www.ncbi.nlm.nih.gov/

```
 781 ggggatggaa ccttccagaa gtgggcggct gtggtggtgc cttctggaca ggagcagaga
 841 tacacctgcc atgtgcagca tgagggtttg cccaagcccc tcaccctgag atgggagccg
 901 tcttcccagc ccaccatccc catcgtgggc atcattgctg gcctggttct ctttggagct
 961 gtgatcactg gagctgtggt cgctgctgtg atgtggagga ggaagagctc agatagaaaa
1021 ggagggagct actctcaggc tgcaagcagt gacagtgccc agggctctga tgtgtctctc
1081 acagcttgta aagtgtga
```

The sequence of β2-m was extracted from ncbi: http://www.ncbi.nlm.nih.gov/

```
ORIGIN
        1 aatataagtg gaggcgtcgc gctggcgggc attcctgaag ctgacagcat tcgggccgag
       61 atgtctcgct ccgtggcctt agctgtgctc gcgctactct ctctttctgg cctggaggct
      121 atccagcgta ctccaaagat tcaggtttac tcacgtcatc cagcagagaa tggaaagtca
      181 aatttcctga attgctatgt gtctgggttt catccatccg acattgaagt tgacttactg
      241 aagaatggag agagaattga aaaagtggag cattcagact tgtctttcag caaggactgg
      301 tctttctatc tcttgtacta cactgaattc acccccactg aaaaagatga gtatgcctgc
      361 cgtgtgaacc atgtgacttt gtcacagccc aagatagtta agtgggatcg agacatgtaa
      421 gcagcatcat ggaggtttga agatgccgca tttggattgg atgaattcca aattctgctt
      481 gcttgctttt taatattgat atgcttatac acttacactt tatgcacaaa atgtagggtt
      541 ataataatgt taacatggac atgatcttct ttataattct actttgagtg ctgtctccat
      601 gtttgatgta tctgagcagg ttgctccaca ggtagctcta ggagggctgg caacttagag
      661 gtggggagca gagaattctc ttatccaaca tcaacatctt ggtcagattt gaactcttca
      721 atctcttgca ctcaaagctt gttaagatag ttaagcgtgc ataagttaac ttccaattta
      781 catactctgc ttagaatttg ggggaaaatt tagaaatata attgacagga ttattggaaa
      841 tttgttataa tgaatgaaac attttgtcat ataagattca tatttacttc ttatacattt
      901 gataaagtaa ggcatggttg tggttaatct ggtttatttt tgttccacaa gttaaataaa
      961 tcataaaact tgatgtgtta tctctta
```

2.4.1 Cloning of the HLA-A*0201 and b-2m gene from PBMC[8]

Protocol:

1. Isolate[9] the total RNA that was extracted from the peripheric blood monocytes (PBMC) from 10 ml human_s anticoagulative venous blood was dissolved in 50μL ddH2O.
   RESULT: total RNA only

2. Quantify by ultraviolet or spectrophotometer. *Goal: for incertitude that find RNA if the number < 1.3.*
3. Amplify The extracellular fragment of HLA-A*0201 (including the fragment of transmembrane) using total RNA as a template with the forward primer: 5′-

[8] This step was extracted from Protein Expression and Purification 35 (2004) 210-21, form *science direct* www.sciencedirect.com.

[9] The procedures of this step as follows in peqGOLD kit and for more details please see the part 3.5.

CCCTGACCCAGACCTGGGCGG-3′ and the reverse primer 3′-AGGGTCGGGTGGTAGGGGTAG-5′.by PCR as follows:

*RESULT: Amplification the DNA segment of HLA-A*0201 only.*

4. Then using the PCR-product as a template amplified the just extracellular fragment of HLA-A*0201 with the forward primer 5′-GGCTCCCACTCCATGAGGTAT-3′ and the reverse primer 3′-GGGAGTGGGACTCTACCCTCG -5′.
   *RESULT: by nested PCR can surely the amplification of DNA segment of HLA-A*0201.*
5. Analogously, we constructed b-2m expression vector; however, there were some differences between them. The fragment encoding b-2m was amplified using total RNA as a template with the forward primer 5′-GCATCCAGCGTACTCCAAAGA-3′ and the reverse primer 3′-ATTCACCCTAGCTCTGTACCG-5′. The PCR protocol was the same as that used in amplification of HLA-A*0201.
   RESULT: Amplification the DNA segment of β2m only.
6. After purification from an agarose gel. The DNA sequences were verified by sequencing.

2.4.2 Expression of HLA-A*0201 and b-2m

We referred on this step to expression by pETvector with BL21 (DE3) (host strain for expression).

2.4.2.1 Prepare pET Vector:

To digest and gel-purify the vector[10]:

Reagent:

- pET vector
- 10X restriction enzyme buffer
- EcoR I restriction enzyme
- calf intestinal alkaline phosphatase

1. Assemble the following components in a microcentrifuge tube:

- 3 µg pET vector
- 3 µl 10X restriction enzyme buffer
- 10–20 U EcoR I restriction enzyme (assuming compatible buffer; the total volume of enzyme added should not exceed 10% of the reaction volume to avoid high glycerol concentrations)
- x µl Nuclease-free water brought to volume
  30 µl Total volume

2. Incubate at the appropriate temperature (usually 37°C) for 2–4 h.

3. Run a 3 µl sample together with Perfect DNA™ Markers on an agarose gel to check the extent of digestion.

---

1. [10] This step from Novagen."
   lifeserv.bgu.ac.il/wb/zarivach/.../Novagen%20**pET**%20**system**%20**manual**.pdf"

4. When digestion is complete, add calf intestinal alkaline phosphatase (Calbiochem Cat. No.524576) directly to the remainder of the digestion. The enzyme functions in most restriction buffers under the conditions described here. It is important to use the correct amount of enzyme; too much can cause unwanted deletions and can be difficult to remove for future steps. Three μg of a typical pET vector (5 kbp) corresponds to about 2 pmol DNA ends when linearized, or about 4 pmol ends if two enzymes were used for digestion. We recommend using 0.05 units of alkaline phosphatase per pmol ends. Dilute the enzyme in water or 50 mM Tris-HCl, pH 9.0 just before use.

5. Incubate at 37°C for 30 min.

6. Add gel sample buffer to the reaction and load the entire sample into a large well (0.5–1.0 cm wide) on a 1% agarose gel containing 0.5 μg/ml ethidium bromide. Run the gel far enough to separate the linear plasmid from nicked and supercoiled species. It is useful to run uncut vector DNA in an adjacent lane to help distinguish undigested from linearized plasmid DNA.

7. Visualize the DNA band with a long wave UV light source and cut the band from the gel using a clean razor blade. Avoid over exposure to the light source, which can cause nicks and double strand breaks in the DNA.

8. Recover the DNA from the gel slice. The SpinPrep™ Gel DNA Kit (Cat. No. 70852-3) is ideal for this application. Resuspend the final product in a total volume of 30 μl (usually about 50 ng/μl DNA). The DNA can be quantified spectophotometrically or using the PicoGreen kit from Molecular Probes. Assume recoveries in the range of 50% for the ligation step.

9. Store the treated vector at –20°C until use.

2.4.2.2 Prepare the insert DNA with the adapter

Suggested Conditions for Adapter Addition to DNA[11].

Reagent:

1. 5X adapter buffer
2. *Eco*R I(*Not* I) adapter (1 mg/ml)
3. 0.1 M DTT
4. T4 DNA ligase (1 U/μl)
5. T4 polynucleotide kinase.

1. Add the following reagents to a microcentrifuge tube on ice.

- Up to 5 μg blunt-ended DNA
- 10 μl 5X adapter buffer (330 mM Tris-HCl (pH 7.6),50 mM MgCl2, 5 mM ATP)
- 10 μl *Eco*R I(*Not* I) adapter (1 mg/ml)[12]

[11] This step was extracted from invitrogen life technologies.

[12] *Eco*R I(*Not* I) adapter may be used to add *Eco*R I cohesive ends directly to any blunt-ended DNA fragment. The adapter is formed by annealing two oligonucleotides together forming a phosphorylated, blunt end and a nonphosphorylated *Eco*R I half-site. Ligation to blunt ended DNA results in *Eco*R I half-sites on both ends of the DNA. The adapted DNA may be ligated into any *Eco*R I containing vector. The adapter also contains the recognition

- 7 µl 0.1 M DTT
- distilled water sufficient to bring the volume to 45 µl.

2. Then add 5 µl of T4 DNA ligase (1 U/µl) and mix gently.

3. Incubate the reaction for a minimum of 16 h at 16°C.

4. Heat the reaction at 70°C for 10 min to inactivate the ligase.

5. Place the reaction on ice. The adapted DNA, after adapter removal (step 9) may be ligated directly to any phosphorylated, *Eco*R I-digested vector.

However, if a dephosphorylated *Eco*R I-digested vector is used, the adapted cDNA must be phosphorylated first before adapter removal (step 6).

6. Add 3 µl (30 units) of T4 polynucleotide kinase to the reaction from step 5.

7. Mix gently, and incubate the reaction for 30 min at 37°C.

8. Heat the reaction at 70°C for 10 min to inactivate the kinase and place the reaction on ice.

9. Remove the excess *Eco*R I(*Not* I) adapters by column chromatography (ie. cDNA Size Fractionation Columns, Cat. No. 18092-015) or by some other method (ie. gel electrophoresis) and then ligate into the appropriate vector.

2.4.2.3 LIGATION

Reagent:

- 10X Ligase Buffer (200 mM Tris-HCl pH 7.6,
- 100 mM MgCl2
- 100 mM DTT
- 10 mM ATP
- 50 ng/µl prepared pET vector
- T4 DNA ligase, diluted (with ligase dilution buffer) 0.2–0.4 Weiss units/µl
- Prepared target gene insert (0.2 pmol)
- Nuclease-free water
- Ligase

Procedures:

One consistently successful protocol for ligation is presented here.

---

sequences for *Not* I and *Sal* I restriction enzymes. Thus, DNA inserts may be cleaved from the vector using *Eco*R I or either of the rare cutting enzymes, *Not* I or *Sal* I.
Adapter sequence: 5'-pGTCGACGCGGCCGCG
CAGCTGCGCCGGCGCTTAA-OH-5'
Storage Buffer
10 mM Tris-HCl (pH 7.5)
100 mM NaCl
1 mM EDTA
Quality Control Assays:
This product has passed a self-ligation quality control assay.
Doc. Rev.: 100901

1. For a standard reaction using DNA fragments with 2–4 base sticky ends, use 50–100 ng (0.015–0.03 pmol) of pET vector with 0.2 pmol insert (50 ng of a 500 bp fragment) in a volume of 20 µl. Assemble the following components in a 1.5 ml tube (these components are available separately in the DNA Ligation Kit, Cat. No. 69838-3) or use the Clonables™ 2X Ligation Premix (Cat. No. 70573-3). Add the ligase last.

- 2 µl 10X Ligase Buffer (200 mM Tris-HCl pH 7.6,
- 100 mM MgCl2
- 2 µl 100 mM DTT
- 1 µl 10 mM ATP
- 2 µl 50 ng/µl prepared pET vector
- 1 µl T4 DNA ligase, diluted (with ligase dilution buffer) 0.2–0.4 Weiss units/µl
- x µl Prepared target gene insert (0.2 pmol)
- y µl Nuclease-free water to volume
  20 µl Total volume

2. Add the ligase last, and gently mix by stirring with a pipet tip. Incubate at 16°C for 2 h to overnight. Also set up a control reaction in which the insert is omitted to check for nonrecombinant background.

Note: For blunt ends, use 10X more ligase (i.e., undiluted enzyme), reduce the ATP concentration to 0.1 mM and incubate for 6–16 h at 16°C or 2 h at room temperature.

Transformation INTO EXPRESSION HOST

Reagent:

- competent cell.
- SOC Medium.
- LB agar plates

Procedures:

1. Remove the appropriate number of competent cell tubes from the freezer (include one extra sample for the Test Plasmid positive control, if desired). Immediately place the tubes on ice, so that all but the cap is surrounded by ice. If the standard cells are to be used, place the required number of empty 1.5 ml polypropylene microcentrifuge tubes on ice to prechill. Allow the cells to thaw on ice for ~2–5 min.
2. Visually check the cells to see that they have thawed and gently flick the cells 1–2 times to evenly resuspend the cells.
3. *Standard Competent Cells*:
Pipet 20 µl aliquots of cells into the pre-chilled tubes.
Singles Competent Cells:
Proceed to Step 4 or 5, depending on whether a Test Plasmid sample is included as a positive control.
4. (Optional) to determine transformation efficiency, add 0.2 ng (1 µl) Test Plasmid provided with Competent Cells to one of the tubes containing cells. Stir gently to mix and return the tube to the ice.
5. Add 1 µl of a ligation reaction or purified plasmid DNA directly to the cells. Stir gently to mix and return the tube to the ice, making sure that the tube is surrounded by ice except for the cap. Repeat for additional samples.

6. Incubate the tubes on ice for 5 min.
7. Place the tubes in a 42°C water bath for exactly 30 sec; do not shake.
8. Place the tubes on ice for 2 min.
9. *Standard Competent Cells:*
Add 80 µl of room temperature SOC Medium to each tube. Keep the tubes on ice until all have received SOC.
Singles Competent Cells
Add 250 µl of room temperature SOC Medium to each tube. Keep the tubes on ice until all have received SOC.
10. Selection for transformation is accomplished by plating on medium containing antibiotic for the plasmid encoded drug resistance. Additional host-specific antibiotics may also be appropriate to ensure maintenance of the host feature(s).
When using NovaBlue: if selecting for β-lactamase (carbR/ampR), no outgrowth (shaking incubation) step is required, although slightly higher cloning efficiencies may be obtained with 30–60 min outgrowth. Plate 5–50 µl cells directly on selective media. If selecting for kanamycin resistance, shake at 37°C (250 rpm) for 30 min prior to plating.
When using strains other than NovaBlue: shake at 37°C (250 rpm) for 60 min prior to plating.
Prepare LB agar plates with appropriate antibiotic ahead of time
Notes: The outgrowth incubation is conveniently performed in a shaking incubator using a test tube rack anchored to the shaking platform. Place each transformation tube in an empty 13 mm x 100 mm glass test tube in the rack. The snap-caps on the transformation tubes prevent them from falling to the bottom of the test tubes, and all transformation tubes remain vertical.
During the outgrowth (or earlier if omitting outgrowth), place the *plates at 37°C. If the plates contain a lot of moisture, place them cover-side up and ope*n the cover ~1/3 of the way to allow the plates to dry for 30–45 min. If the plates do not need drying, keep them closed and place them cover-side down in the 37°C incubator for ~20 min prior to plating.
11. Spread 5–50 µl of each transformation on LB agar plates containing the appropriate antibiotic for the plasmid and host strain. When plating less than 25 µl, first pipet a "pool" of SOC onto the plate and then pipet the cells into the SOC. Please see the part 4.6 for additional details on plating technique.
Important: The appropriate amount of transformation mixture to plate varies with the efficiency of both the ligation and the competent cells. As little as 2 µl will yield several hundred transformants under highly efficient conditions (e.g., with NovaBlue cells giving > 4 × 10 8 cfu/µg). For recombinants in NovaBlue, expect 10 5–10 7 transformants/µg plasmid, depending on the particular insert and the ligation efficiency.
When using the Test Plasmid, plate no more than 5 µl (e.g., 5 µl of NovaBlue cells at 1 × 10 8 efficiency) or 10 µl (e.g., 10 µl of cells at 1 × 10 6 efficiency) of the final transformation mix in a pool of SOC on an LB agar plate containing 50 µg/ml carbenicillin or ampicillin (because the Test Plasmid carries the ampR gene).
12. Let the plates sit on the bench for several min to allow excess liquid to be absorbed, and then invert and incubate overnight at 37°C.

2.4.3 Extraction and solubilization of inclusion bodies

Reagent:

- 10mM tris HCl , pH8
- 10 mM tris HCl, pH7

- 8M Urea, 100 mM Tris HCl, pH8
- 8M urea 20 mM Tris ,PH 8
- LYSOSYM (100 µg /ml)
- Phenyl methyl sulfonyl fluorid (50µg/ml)
- DNase (20µg/ml)
- RNase (20µg/ml)
- 1mM EDTA
- 0-100 mM NaCl

Purification of recombinant proteins:

1- The cell were harvested by centrifugation at an OD650 OF 1.8-2.0
2- The cell pellets were resuspended in 10 mM Tris HCl.pH 8 (20 ml) resuspension, containing lysozyme (100 µg / ml), phenyl methyl sulfonyl fluoride (50 µg / ml), DNase(20 µg/ml),RNase (20 µg / ml),and 1mM EDTA and incubated at 22◦c for 20 min.
3- The cells were lysed by sonication[13] and then centrifuged (10000xg).for 20 min.
4- The pellet containing recombinant protein was washed with 10 mM Tris HCl, pH 8(20 ml).
5- It is then dissolved in 100 mM Tris HCl, pH8/8 M urea (10 ml), and centrifuged at 4°C for 1 hour.(150000xg)
6- The recombinant protein purified on Q Sepharose fast flow[14].

Using a HiTrap 1-ml column with a syringe. **(A)** Prepare buffers and sample. Remove the column's top cap and snap off the end. Wash and equilibrate. **(B)** Load the sample and begin collecting fractions. **(C)** Wash, elute, and continue collecting fractions[15].

7- fractions, the purified HLA heavy chains contained stored at-20°C for use in the ELISA experiment

2.4.4 ELISA assay of peptide-MHC complex formation:

Material:

[13] **Sonication** is the act of applying sound (usually ultrasound) energy to agitate particles in a sample.

In biological applications, sonication may be sufficient to disrupt or deactivate a biological material. This process is called sonoporation. Sonication is also use to fragment molecules of DNA. This is an alternative to the freeze-pump-thaw(**by Schlenk flask**) and sparging methods. It is especially useful when it is not possible to stir the sample, as with NMR tubes

[14] The ion exchange chromatography Q sepharose FF was purchased from GE Healthcare. see www.gelifesciences.com/hitrap for more details

[15] Took from GE Healthcare.

- Pan specific mouse anti HLA class I antibody,W6/32
- Polyclonal rabbit antihuman β2m-HRP
- dextran polymer conjugated with goat antirabbit IgG and HRP
- 96 well Maxisorp ELISA Plates
- Pluronic lutrol F-68
- Peptide
- 100mM carbonate buffer (PH 9.6)
- 10٪ w/v skimmed milk powder in PBS (SMP-PBS)
- 0.05٪ Tween-20 in PBS
- 0.3 mM Tris-maleat buffer(PH 6.6)

Day 1

-96 well Maxisorp ELISA Plates were coated and let overnight at 4°C with W6/32.

Using 50 µl/well at 5 µl/ml, in 100 mM carbonate buffer, PH 9.6.

Day 2

-Add 320 µl/ well 10٪ w/v skimmed milk powder in PBS (SMP-PBS) to block the residual bind.

-Wash twice with 600 µl/ well of 0.05 ٪ Tween-20 in PBS at room temperature using an automated plate washer to remove unbound W6/32 and blocking reagent

-On ice, purified recombinant HLA molecule in 8M Urea and 20mM tris PH8 were diluted 100-fold into a 0.3 mM tris maleat buffer, PH 6.6, contaning human β2m, peptide and lutrol F- 68 at the concentrations indicated.

3nM MHC-I HC is optimal

1g/L lutrol F-68

100nM β2m is optimal

10,000nM petid (optimal 1nM to 1µM)

- To allow complex formation the reaction mixtures were incubated at 18°C for 48h.

Day 3

Incubation

Day 4

-Just prior to the ELISA analysis, the reaction volume was diluted 10 times into 2٪ SMP/PBS at 4°C.

-50 µl/well with PBS/0.05% Tween 20 were transferred in triplicate to a W6/32 coated plate.

-The plate was incubated for 2h at 4°C.

-Washed 6 times with 600 µl/well with PBS/0.05% Tween 20 at room temperature.

-detect the binding complex,the plate was incubated for 1h at 4°c , with 50 µl/ well of a polyclonal rabbit antihuman β2m-HRP diluted 1:2500 into 2% SMP-PBS and the washed 6*600 µl/well with PBS 0.05% tween 20 at room temperature

-To enhance the detection, the plate was subsequently incubated for 30 min at room temperature with a dextran polymer conjugated with goat antirabbit IgG and HRP diluted in 1:15 in 2% SMP-PBS containing 1% normal mouse serum.

-Washed 6*600 µl/well with PBS/0.05% Tween 20 at room temperature.

-ELISA was developed with 3,3′ 5,5′-tetramethylbenzidine hydrogenperoxide for 30 min at room temperature.

-Colorimetric reaction was read at 450nm using a Victor Multilabel ELISA counter

2.4.5 More details:

I- The peqGOLD total RNA Kit

INTRODUCTION

The peqGOLD Total RNA Kit provides a rapid and easy method for the isolation of up to 100 μg of total RNA from eukaryotic cells and tissues. This kit allows processing of a single or multiple samples in less than 30 min. Normally, up to 1 x 107 cells or 40 mg tissue can be used in a single experiment. There is no need for phenol/chloroform extractions and time-consuming steps such as CsCl gradient ultracentrifugation or precipitation with isopropanol. While this kit may be used for isolation of RNA from whole blood, we recommend to use the peqGOLD Blood RNA Kit (product # 12-6814) as it is specifically designed for effective hemolysis and hemoglobin removal and gives therefore higher RNA yields from blood.

RNA purified using the peqGOLD Total RNA Kit is ready for applications such as RT-PCR, Northern blotting, poly(A)+-RNA (mRNA) purification, nuclease protection assays and in vitro translation.

peqGOLD Total RNA Kits are available with Safety-Line (Order No. 12-6834-xx) or Classic Line (Order No. 12-6634-xx) columns. Safety-Line columns can be closed tightly by lids to avoid cross-contamination more effectively. Classic-Line colums do not have lids for a more comfortable handling.

PRINCIPLE

The peqGOLD Total RNA Kit uses the reversible binding properties of the PerfectBind RNA Column, a new silica-based material. This is combined with the speed of minicolumn spin technology. A specifically formulated high salt buffer system allows more than 100 μg of RNA molecules greater than 200 bases to bind to the matrix. Cells or tissues are first lysed under denaturing conditions that practically inactivate RNases. Samples are then applied to the PerfectBind RNA Columns to which total RNA binds, while cellular debris and other contaminants are effectively washed out. High quality RNA is finally eluted in RNase-free sterile water.

KIT COMPONENTS

peqGOLD Total RNA Kit
Order No. Safety-Line
Order No. Classic-Line
Components
PerfectBind RNA Columns
DNA Removing Columns
2.0 ml Collection Tubes
RNA Lysis Buffer T
RNA Wash Buffer I
RNA Wash Buffer II (conc.)
RNase-free Water
Instruction manual

STORAGE AND STABILITY

The peqGOLD Total RNA Kit components should be stored at room temperature. If stored under these conditions, all components are stable for at least 12 months from the date of purchase. During shipment crystals may form in the RNA Lysis Buffer T. Warm up to 37°C to dissolve.

BEFORE STARTING

Briefly examine this booklet and become familiar with each step. Prepare all components and have the necessary materials ready before starting.

! Whenever working with RNA, always wear one-way gloves to minimize RNase contamination. Use only fresh RNase-free disposable plastic pipette tips when using the supplied reagents.

! Work carefully but as quickly as possible during the procedure.

! Under cool ambient conditions, crystals may form in RNA Lysis Buffer T. This is normal and the bottle should be warmed (37 °C) to dissolve the salt before use.
! RNA Wash Buffer II is concentrated and has to be diluted with absolute ethanol as follows:
12-6834-00 Add 8 ml 100% EtOH to 2 ml Wash Buffer II.
12-6834-01 Add 80 ml 100% EtOH to 20 ml Wash Buffer II.
12-6834-02 Add 3 x 80 ml 100% EtOH to 3 x 20 ml Wash Buffer II.
! Store diluted RNA Wash Buffer II at room temperature.
! All steps must be carried out at room temperature (22 – 25 °C).

PEQGOLD TOTAL RNA ISOLATION PROTOCOL

Eucaryotic cells and tissue

Materials to be supplied by the user:
! 100 % Ethanol
! 70 % Ethanol in sterile RNase-free dH2O
! Sterile RNase-free pipet tips and centrifuge tubes

1. Homogenization and lysis

a. Tissue

Excise tissue (~ 40 mg, 3 mm3) and promptly freeze in a small volume of liquid nitrogen.
Grind tissue with a ceramic mortar and pestle under approximately 10 ml of liquid nitrogen.
Wear gloves and take great care when working with liquid nitrogen.
Transfer the suspension into a pre-cooled 15 ml polypropylene tube. If the tube is not precooled (in liquid nitrogen), the suspension will boil vigorously possibly causing loss of tissue. When the liquid nitrogen has completely evaporated, add 400 µl RNA Lysis Buffer T. Transfer the lysate directly into a DNA Removing Column placed in a 2.0 ml Collection Tube. Centrifuge at 12.000 x g for 1 min at room temperature. Transfer the flow-through lysate into a new 1.5 ml tube.
For RNase rich tissues or more than 40 mg tissue, use 600 µl of RNA Lysis Buffer T. However, do not use more than 50 mg tissue.
For homogenization, you may also use glass-, teflon- or electric homogenisators.

b. Monolayer Cells

For tissue culture cells grown in monolayer (adherent fibroblasts, endothelial cells etc.), lyse the cells directly in the culture vessel as follows. Aspirate culture medium completely and add RNA Lysis Buffer T directly to the cells. Use 800 µl for T35 flasks or 10 cm dishes, and 400 µl for smaller vessels. Pipet buffer over entire surface of vessel to ensure complete lysis. Transfer the lysate directly into a DNA Removing Column placed in a 2.0 ml Collection Tube. Centrifuge at 12.000 x g for 1 min at room temperature. Transfer the flow-through lysate into a new 1.5 ml tube.
This method is preferable to trypsinization followed by washing because it minimizes RNA degradation by nuclease contamination.

c. Suspension culture

For cells grown in suspension cultures, pellet cells at 1.500 rpm (400 x g) for 5 min. Pour off supernatant and add 400 µl RNA Lysis Buffer T per 1 x 107 cells. Transfer the lysate directly into a DNA Removing Column placed in 2.0 ml Collection Tube. Centrifuge at 12.000 x g for 1 min at room temperature. Transfer the flow-through lysate into a new 1.5 ml tube.

2. Load and Bind

Add an equal volume (400 µl, 600 µl or 800 µl) 70 % Ethanol to the lysate and mix thoroughly by vortexing. Place a PerfectBind RNA Column in a new 2.0 ml Collection Tube (supplied) and add the lysate directly to the membrane. Centrifuge the PerfectBind RNA Column / Collection Tube assembly at 10.000 x g for 1 min. Discard the flowthrough liquid and Collection Tube.

A precipitate may form on addition of 70 % ethanol. Vortex and add the entire mixture to the column. The maximum capacity of the PerfectBind RNA Column is 750 µl, larger volumes can be loaded successively. However, the total binding capacity of a PerfectBind RNA Column is approx.
100 µg RNA.

3. Wash I

Place the PerfectBind RNA Column in a fresh 2.0 ml Collection Tube, add 500 µl RNA Wash Buffer I to the PerfectBind RNA Column and centrifuge for 15 sec at 10.000 x g. (supplied). Discard the flow-throw liquid and reuse the collection tube in the next step.

4. DNase Digestion (optional)

Since PerfectBind RNA Column technology actually removes most of DNA without a DNase treatment, it is not necessary to do DNase digestion for most downstream applications. However, certain sensitive RNA applications might require further DNA removal. Following steps provide on-membrane DNase I digestion (Order No. 12-1091).

a. For each PerfectBind RNA Column, prepare this DNase I digestion reaction mix:
DNase I Digestion Buffer 73.5 µl, RNase-free DNase I (20 Kunitz units/µl) 1.5 µl, Total volume 75.0 µl.

Note:
1. DNase I is very sensitive to physical denaturation, so do not vortex this DNase I mixture!
Mix gently by inverting the tube.
Prepare fresh DNase I digestion mixture directly before RNA isolation.
2. DNase I digestion buffer is supplied with RNase-free DNase set.
Standard DNase buffers are not compatible with on-membrane DNase digestion!

b. Pipet 75 µl of the DNase I digestion reaction mix directly onto the surface of PerfectBind RNA resin in each column. Make sure to pipet the DNase I digestion mixture directly onto the membrane.
DNase I digestion will not be complete if some of the mix stick to the wall or the O-ring of the PerfectBind RNA Column.

c. Incubate at room temperature (25 – 30 °C) for 15 minutes.

d. Place the PerfectBind RNA Column into a 2.0 ml Collection Tube and add 400 µl RNA
Wash Buffer I. Incubate the PerfectBind RNA Column at benchtop for 5 minutes.
Centrifuge at 10.000 x g for 5 minutes and discard flow-through. Re-use collection tube in the next step. Continue with step 5.

5. Wash II

Add 600 µl completed RNA Wash Buffer II to the PerfectBind RNA Column and centrifuge for 15 sec at 10.000 x g. Discard the flow-through liquid. Repeat this wash step and discard the flow-through liquid.

6. Dry (Important! Do not skip this step!)

Place the PerfectBind RNA Column containing your RNA in the collection tube used in step 5 and centrifuge for 1 min at 10.000 x g to completely dry the column matrix. This step is essential to remove ethanol from the column.

7. Elution

Place the PerfectBind RNA Column (step 6) into a fresh 1.5 ml microcentrifuge tube. Add 50 - 100 µl (depending on the desired final concentration of RNA) sterile RNAse-free dH2O directly to the binding matrix in the PerfectBind RNA Column and centrifuge for 1min at 5.000 x g to elute RNA.
A second elution may be necessary if the expected yield of RNA is > 50 µg. Alternatively, RNA may be eluted with a higher volume of water. While an additional elution increase total RNA yield, the concentration will be lowered since more than 80 % of RNA is recovered with the first elution. Pre-heating

RNase-free dH2O to 70 °C before adding to the PerfectBind RNA Column and incubating the PerfectBind RNA Column for 5 min at room temperature before centrifugation may increase yield.

DNA CONTAMINATION

No RNA extraction procedure can completely remove genomic DNA. For sensitive work (such as RT-PCR or differential display) we suggest that you treat the eluted RNA with RNase-free DNase. On-membrane DNase I Digestion is a simple and fast method and can be integrated into the standard protocol between the washing steps (see page 14/15).

Also for RT-PCR, use intron-spanning primers that allow easy identification of DNAcontamination.

A PCR reaction, which uses the RNA as template, will also allow the detection of DNA contamination.

QUANTITATION AND STORAGE OF RNA

Determine the absorption of an appropriate dilution (10- to 50-fold) of the sample at 260 nm and 280 nm.

RNase-free water is slightly acidic and can dramatically lower absorption values. We suggest that you dilute the sample in a buffered solution (TE) for spectrophotometric analysis.

One A260-unit is about 40 µg RNA/ml. The RNA concentration is calculated as follows:

RNA conc. (µg /ml) = Absorption260 × 40 × Dilution Factor

The ratio of A260/280 is an indication of nucleic acid purity. A value higher than 1.8 indicates > 90% nucleic acid.

Store RNA samples at – 70 oC in sterile RNase-free dH2O. Under such conditions RNA prepared with the peqGOLD system is stable for at least one year.

RNA QUALITY

It is highly recommended to determine the RNA quality prior to further applications.

Denaturing agarose gel electrophoresis and ethidium bromide staining can best assess the quality of RNA. Two sharp bands should appear on the gel. These represent the 28S and 18S ribosomal RNA bands. If these bands smear towards lower molecular weight RNA, then the RNA has undergone major degradation during preparation, handling or storage.

Although RNA molecules less than 200 bases in length do not efficiently bind to the PerfectBind RNA Column, a third RNA band, the tRNA band, may be visible when a large number of cells are used.

B- Part II

The protocol that will be practiced in MEGBI lab contains many modifications for the protocol of part I to fit with the materials and the devices presented in the lab.

2.5 Amplification of the HLA-A*0201 and β-2m gene from PBMC

Reagent:

peqGOLD kit.
Forward primer for HLA-A*0201: 5′-CCCTGACCCAGACCTGGGCGG-3′
Reverse primer for HLA-A*0201: 3′-AGGGTCGGGTGGTAGGGGTAG-5′
Forward primer 3 for HLA-A*0201: 5′-GGCTCCCACTCCATGAGGTAT-3′
Reverse primer 4 for HLA-A*0201: 3′-GGGAGTGGGACTCTACCCTCG -5′.
Forward primer for β2-m: 5′-GCATCCAGCGTACTCCAAAGA-3′
Reverse primer for β2-m: 3′-ATTCACCCTAGCTCTGTACCG-5′
dNTP Mix
Sterile, distilled water
M DTT
(200 units) of M-MLV (RT)
RNase A
10XPCR Buffer [200mM Tris-HCl (PH 8.4), 500 mM KCl]
50 mM $MgCl_2$
10 mM dNTP Mix
Amplification primer 1(10μM)
Amplification primer 2 (10μM)
Taq DNA/polymerase

Protocol:

1. Isolate[16] the total RNA that was extracted from the peripheric blood monocytes (PBMC) from 10 ml human_s anticoagulative venous blood was dissolved in 50μL ddH2O.

RESULT: the total RNA only

2. Quantify by ultraviolet or spectrophotometer. *Goal: for incertitude that find RNA if the number < 1.3.*

3. Amplify the extracellular fragment of HLA-A*0201 (including the fragment of transmembrane) using total RNA as a template with the forward primer: 5′-CCCTGACCCAGACCTGGGCGG-3′ and the reverse primer 3′-AGGGTCGGGTGGTAGGGGTAG-5′.by PCR as follows:

[16] The procedures of this step as follows in peqGOLD kit and for more details see the last page.

a. Add the following components to a nuclease-free microcentrifuge tube:

- Take 0.6µl of the Primer.

- Take 5µl of the total RNA.

- 1µl (10mM) dNTP Mix (10mM each dATP,dGTP,dCTP and dTTP at neutral PH).

- Add sterile, distilled water to 12 µl.

b. Heat mixture to 65°C for 5 min and quick chill on ice.

C. collect the content of the tube by brief centrifugation (e.g. 20s , 10 000 rpm)

And add:

- 4µl 5X First-Strand Buffer

- 2µl 0.1 M DTT[17]

c. Mix contents of the tube gently and incubate at 37 °C for 2 min

d. Add 1µl (200 units) of M-MLV (RT)[18] , and mix by pipetting gently up and down.

e. Incubate 50 min at 37°C.

f. Inactivate the reaction by heating at 70°C for 15 min.

g. Incubate 40s at 90 °C (with PCR machine)

h. Immediately put the tube into ice for 1 min

i. Add 3µl RNase A

j. Incubate 20 min at 37°C (with PCR machine)

Now we have cDNA.

4-Add the above to a PCR reaction tube for a final reaction volume of 50 µl:

- 2.5µl 10×Reaction buffer S
- 2.5µl 10×Reaction buffer Y
- 10µl enhancer solution
- 1µl 40 mM dNTP Mix
- 1µl amplification primer 1(10µM)
- 1µl amplification primer 2 (10µM)
- 0.5 µl Taq DNA/polymerase (5U/µl)
- 2µl cDNA (from first-strand reaction)
- Autoclaved, distilled water to 50 µl

[17] DTT, Molecular Grade, is an antioxidant used to stabilize enzymes and other proteins containing sulfhydryl groups. The liquid form of the product is a 100mM solution of DTT in water(Promega)

[18] take the tube of M-MLV out and put in ice until thawed

- Mix gently and layer 1-2 drops (50µl) of silicone oil over the reaction
- (Note: the addition of silicone oil is unnecessary in thermal cyclers equipped with a heated lid.)
- Heat reaction to 94°C for 2 min to denature.
- PCR program: 35 cycles:
- 10s 95 °C
- 10s 60 °C
- 30-60s 72 °C
- Take 5µl from the result and put in the second round PCR and repeat the same step.

(Note: we use inner primers 3 and 4 in the second round of PCR)

5-Put the PCR product on the gel agarose.

*RESULT: Amplification the DNA segment of HLA-A*0201 only.*

Analogously, we constructed β-2m expression vector; however, there were some differences between them. The fragment encoding β -2m was amplified using total RNA as a template with the forward primer 5′-GCATCCAGCGTACTCCAAAGA-3′ and the reverse primer 3′-ATTCACCCTAGCTCTGTACCG-5′. The PCR protocol was the same as that used in amplification of HLA-A*0201.

RESULT: Amplification the DNA segment of β2m only.

Purify from an agarose gel by QIA quick gel extraction kit[19] . The DNA sequences were verified by sequencing.

2.5.1 Prepare the insert DNA (HLA-A*0201 and *β2m*) with the adapter

Suggested Conditions for Adapter Addition to DNA[20].

Reagent:

5X adapter buffer

*Eco*R I(*Not* I) adapter (1 mg/ml)

0.1 M DTT

T4 DNA ligase (1 U/µl)

T4 polynucleotide kinase.

1. Add the following reagents to a microcentrifuge tube on ice.

Up to 5 µg blunt-ended DNA

10 µl 5X adapter buffer (330 mM Tris-HCl (pH 7.6),50 mM MgCl2, 5 mM ATP)

[19] See MEGBI training courses book part I page 54-58

[20] This step was extracted from invitrogen life technologies.

10 µl *Eco*R I(*Not* I) adapter (1 mg/ml)[21]
7 µl 0.1 M DTT
distilled water sufficient to bring the volume to 45 µl.
2. Then add 5 µl of T4 DNA ligase (1 U/µl) and mix gently.
3. Incubate the reaction for a minimum of 16 h at 16°C.
4. Heat the reaction at 70°C for 10 min to inactivate the ligase.
5. Place the reaction on ice. The adapted DNA, after adapter removal (step 9) may be ligated directly to any phosphorylated, *Eco*R I-digested vector.
However, if a dephosphorylated *Eco*R I-digested vector is used, the adapted cDNA must be phosphorylated first before adapter removal (step 6).
6. Add 3 µl (30 units) of T4 polynucleotide kinase[22] to the reaction from step 5.
7. Mix gently, and incubate the reaction for 30 min at 37°C.
8. Heat the reaction at 70°C for 10 min to inactivate the kinase and place the reaction on ice.
9. Remove the excess *Eco*RI (*Not*I) adapters by gel electrophoresis and then ligate into the appropriate vector.

Prepare the T7 RNA polymerase with the adapter

The protocol was the same as that used in preparation of insert DNA with the adapter.

2.5.2 Expression of HLA-A*0201 and β-2m

2.5.2.1 Vector preparation:

To digest and gel-purify the vector:

Reagent:

[21] *Eco*R I(*Not* I) adapter may be used to add *Eco*R I cohesive ends directly to any blunt-ended DNA fragment. The adapter is formed by annealing two oligonucleotides together forming a phosphorylated, blunt end and a nonphosphorylated *Eco*R I half-site. Ligation to blunt ended DNA results in *Eco*R I half-sites on both ends of the DNA. The adapted DNA may be ligated into any *Eco*R I containing vector. The adapter also contains the recognition sequences for *Not* I and *Sal* I restriction enzymes. Thus, DNA inserts may be cleaved from the vector using *Eco*R I or either of the rare cutting enzymes, *Not* I or *Sal* I.
Adapter sequence: 5'-pGTCGACGCGGCCGCG
CAGCTGCGCCGGCGCTTAA-OH-5'
Storage Buffer
10 mM Tris-HCl (pH 7.5)
100 mM NaCl
1 mM EDTA
Quality Control Assays:
This product has passed a self-ligation quality control assay.
Doc. Rev.: 100901

[22] T4 Polynucleotide Kinase (T4 PNK) catalyzes the transfer of the γ-phosphate from ATP to the 5′-terminus of polynucleotides or to mononucleotides bearing a 3′-phosphate group (1). T4 PNK is widely used to end-label short oligonucleotide probes (2), DNA (3) and RNA (4) molecules (Promega)

pET vector
10X restriction enzyme buffer
EcoR I restriction enzyme
1. Assemble the following components in a microcentrifuge tube:
3 µg pET vector
3 µl 10X restriction enzyme buffer
10–20 U EcoR I restriction enzyme (assuming compatible buffer; the total volume of enzyme added should not exceed 10% of the reaction volume to avoid high glycerol concentrations)
x µl Nuclease-free water brought to volume
30 µl Total volume
2. Incubate at the appropriate temperature (usually 37°C) for 2–4 h.
3. Run a 3 µl sample on an agarose gel to check the extent of digestion.
4. When digestion is complete, add calf intestinal alkaline phosphatase (Calbiochem Cat. No.524576) directly to the remainder of the digestion. The enzyme functions in most restriction buffers under the conditions described here. It is important to use the correct amount of enzyme; too much can cause unwanted deletions and can be difficult to remove for future steps. Three µg of a typical pET vector (5 kbp) corresponds to about 2 pmol DNA ends when linearized, or about 4 pmol ends if two enzymes were used for digestion. We recommend using 0.05 units of alkaline phosphatase per pmol ends. Dilute the enzyme in water or 50 mM Tris-HCl, pH 9.0 just before use.
5. Incubate at 37°C for 30 min.
6. Add gel sample buffer to the reaction and load the entire sample into a large well (0.5–1.0 cm wide) on a 1% agarose gel containing 0.5 µg/ml ethidium bromide. Run the gel far enough to separate the linear plasmid from nicked and supercoiled species. It is useful to run uncut vector DNA in an adjacent lane to help distinguish undigested from linearized plasmid DNA.
7. Visualize the DNA band with a long wave UV light source and cut the band from the gel using a clean razor blade. Avoid over exposure to the light source, which can cause nicks and double strand breaks in the DNA.
8. Recover the DNA from the gel slice by purification with QIA quick gel extraction kit[23] Resuspend the final product in a total volume of 30 µl (usually about 50 ng/µl DNA). Assume recoveries in the range of 50% for the ligation step.
9. Store the treated vector at –20°C until use.

2.5.2.2 Ligation

N.B: this step should be done in 3 eppendorfs tubes for HLA-A*0201, β2-m and T7 RNA polymerase.
Reagent:
10X Ligase Buffer (200 mM Tris-HCl pH 7.6,
100 mM MgCl2

[23] Refer to MEGBI training courses book part I page 54-58.

100 mM DTT
10 mM ATP, (or 2× rapid ligation buffer contains ATP)
50 ng/µl prepared pET vector
T4 DNA ligase, diluted (with ligase dilution buffer) 0.2–0.4 Weiss units/µl
Prepared target gene insert (0.2 pmol)
Nuclease-free water

Procedures:

1. For a standard reaction using DNA fragments with 2–4 base sticky ends, use 50–100 ng (0.015–0.03 pmol) of pET vector with 0.2 pmol insert (50 ng of a 500 bp fragment) in a volume of 20 µl. Assemble the following components in a 1.5 ml tube:
2 µl 10X Ligase Buffer (200 mM Tris-HCl pH 7.6,
100 mM MgCl2
2 µl 100 mM DTT
1 µl 10 mM ATP
2 µl 50 ng/µl prepared pET vector
1 µl T4 DNA ligase, diluted (with ligase dilution buffer) 0.2–0.4 Weiss units/µl
x µl Prepared target gene insert (0.2 pmol)
y µl Nuclease-free water to volume
20 µl Total volume
2. Gently mix by stirring with a pipet tip. Incubate at 16°C for 2 h to overnight. Also set up a control reaction in which the insert is omitted to check for nonrecombinant background.

2.5.2.3 Transformation

Preparation of E.coli competent cells[24].

Reagent:

Competent cells JM109.
LB Medium.
LB agar plates.

Procedures:

1. Remove the appropriate number of competent cell tubes from the freezer (include one extra sample for the Test Plasmid positive control, if desired). Immediately place the tubes on ice, so that all but the cap is surrounded by ice. If the standard cells are to be used, place the required number of empty 1.5 ml polypropylene microcentrifuge tubes on ice to prechill. Allow the cells to thaw on ice for ~2–5 min.
2. Visually check the cells to see that they have thawed and gently flick the cells 1–2 times to evenly resuspend the cells.
3. *Standard Competent Cells*:

[24] Refer to MEGBI training courses book part I page 62.

Pipet 20 µl aliquots of cells into the pre-chilled tubes.

Singles Competent Cells:

Proceed to Step 4 or 5, depending on whether a Test Plasmid sample is included as a positive control.

4. (Optional) to determine transformation efficiency, add 0.2 ng (1 µl) Test Plasmid provided with Competent Cells to one of the tubes containing cells. Stir gently to mix and return the tube to the ice.

5. Add 1 µl of each ligation reaction (ligation of HLA-A*0201 and ligation of T7 RNA polymerase) or purified plasmid DNA directly to the cells. Stir gently to mix and return the tube to the ice, making sure that the tube is surrounded by ice except for the cap. Repeat for additional samples.

6. Incubate the tubes on ice for 5 min.

7. Place the tubes in a 42°C water bath for exactly 30 sec; do not shake.

8. Place the tubes on ice for 2 min.

9. *Standard Competent Cells:*

Add 80 µl of room temperature LB medium to each tube. Keep the tubes on ice until all have received LB.

Singles Competent Cells

Add 250 µl of room temperature LB Medium to each tube. Keep the tubes on ice until all have received LB.

10. Selection for transformation is accomplished by plating on medium containing antibiotic (ampicillin) for the plasmid encoded drug resistance.

When using strains other than NovaBlue: shake at 37°C (250 rpm) for 60 min prior to plating.

Prepare LB agar plates with appropriate antibiotic ahead of time

Notes: The outgrowth incubation is conveniently performed in a shaking incubator using a test tube rack anchored to the shaking platform. Place each transformation tube in an empty 13 mm x 100 mm glass test tube in the rack. The snap-caps on the transformation tubes prevent them from falling to the bottom of the test tubes, and all transformation tubes remain vertical.

During the outgrowth (or earlier if omitting outgrowth), place the *plates at 37°C. If the plates contain a lot of moisture, place them cover-side up and ope*n the cover ~1/3 of the way to allow the plates to dry for 30–45 min. If the plates do not need drying, keep them closed and place them cover-side down in the 37°C incubator for ~20 min prior to plating.

11. Spread 5–50 µl of each transformation on LB agar plates containing the appropriate antibiotic for the plasmid and host strain. Please see the last page for additional details on plating technique.

Important: The appropriate amount of transformation mixture to plate varies with the efficiency of both the ligation and the competent cells. As little as 2 µl will yield several hundred transformants under highly efficient conditions (e.g., with NovaBlue cells giving > 4 × 10 8 cfu/µg).

When using the Test Plasmid, plate no more than 5 µl (e.g., 5 µl of NovaBlue cells at 1 × 10 8 efficiency) or 10 µl (e.g., 10 µl of cells at 1 × 10 6 efficiency) of the final transformation mix in a pool of LB on an LB agar plate containing 50 µg/ml carbenicillin or ampicillin (because the Test Plasmid carries the ampR gene).

12. Let the plates sit on the bench for several min to allow excess liquid to be absorbed, and then invert and incubate overnight at 37°C.

2.5.3 Extraction and solubilization of inclusion bodies

Material:

10mM tris HCl , pH8

10 mM tris HCl, pH7

8M Urea, 100 mM Tris HCl, pH8

8M urea 20 mM Tris, PH 8

LYSOSYM (100 μg /ml)

Phenyl methyl sulfonyl fluoride (50μg/ml)

DNase (20μg/ml)

RNase (20μg/ml)

1mM EDTA

0-100 mM NaCl

Purification of recombinant proteins:

The cell were harvested by centrifugation at an OD650 OF 1.8-2.0

The cell pellets were resuspended in 10 mM Tris HCl.pH 8 (20 ml) resuspension, containing lysozyme (100 μg / ml), phenyl methyl sulfonyl fluoride (50 μg / ml), DNase(20 μg/ml),RNase (20 μg / ml),and 1mM EDTA and incubated at 22◦c for 20 min.

The cells were lysed by sonication[25] and then centrifuged (10000xg).for 20 min.

The pellet containing recombinant protein was washed with 10 mM Tris HCl, pH 8(20 ml).

It is then dissolved in 100 mM Tris HCl, pH8/8 M urea (10 ml), and centrifuged at 4°C for 1 hour.(150000xg)

The recombinant protein purified by an ion exchange chromatography Q Sepharose fast flow[26].

fractions, the purified HLA heavy chains contained stored at-20°C for use in the ELISA experiment

2.5.4 ELISA assay of peptide-MHC complex formation:

Material:

- Pan specific mouse anti HLA class I antibody, W6/32
- Polyclonal rabbit antihuman β2m-HRP or post primary block (from Novolink compact polymer Detection kit)

[25] **Sonication** is the act of applying sound (usually ultrasound) energy to agitate particles in a sample. In biological applications, sonication may be sufficient to disrupt or deactivate a biological material. This process is called sonoporation. Sonication is also use to fragment molecules of DNA. This is an alternative to the freeze-pump-thaw (**by Schlenk flask**) and sparging methods. It is especially useful when it is not possible to stir the sample, as with NMR tubes

[26] The ion exchange chromatography Q sepharose FF was purchased from GE Healthcare. see www.gelifesciences.com/hitrap for more details

- dextran polymer conjugated with goat antirabbit IgG and HRP or Novolink polymer **(**from Novolink compact polymer Detection kit**)**
- 96 well Maxisorp ELISA Plates
- Pluronic lutrol F-68
- Peptide
- 100mM carbonate buffer (PH 9.6)
- 10٪ w/v skimmed milk powder in PBS (SMP-PBS) or Protein block (from Novolink compact polymer Detection kit).
- 0.05٪ Tween-20 in PBS
- 0.3 mM Tris-maleat buffer (PH 6.6)

Day 1

-96 well Maxisorp ELISA Plates were coated and let overnight at 4°C with W6/32.

Using 50µl/well at 5µl/ml, in 100 mM carbonate buffer, PH 9.6.

Day 2

Add 320 µl/ well 10٪ w/v protein block to block the residual bind.

Wash twice with 600 µl/ well of 0.05 ٪ Tween-20 in PBS at room temperature using an automated plate washer to remove unbound W6/32 and blocking reagent

On ice, purified recombinant HLA molecule in 8M Urea and 20mM Tris PH8 were diluted 100-fold into a 0.3 mM Tris maleat buffer, PH 6.6, containing human β2m, peptide and lutrol F- 68 at the concentrations indicated:

3nM MHC-I HC is optimal

1g/L lutrol F-68

100nM β2m is optimal

10,000nM peptide (optimal 1nM to 1µM)

To allow complex formation the reaction mixtures were incubated at 18°C for 48h.

Day 3

Incubation

Day 4

Just prior to the ELISA analysis, the reaction volume was diluted 10 times into 2٪ protein block at 4°C.

50 µl/well with PBS/0.05% Tween 20 were transferred in triplicate to a W6/32 coated plate.

The plate was incubated for 2h at 4°C.

Washed 6 times with 600 µl/well with PBS/0.05% Tween 20 at room temperature.

detect the binding complex, the plate was incubated for 1h at 4°c , with 50 µl/ well of a post primary bock with 2% protein block and the washed 6*600 µl/well with PBS 0.05% Tween 20 at room temperature

To enhance the detection, the plate was subsequently incubated for 30 min at room temperature with. Novolink polymer.

Washed 6*600 µl/well with PBS/0.05% Tween 20 at room temperature.

ELISA was developed with 3, 3´ 5, 5´-tetramethylbenzidine hydrogenperoxide[27] for 30 min at room temperature.

Colorimetric reaction was read at 450nm using a Victor Multilabel ELISA counter.

2.6 For more details:

The peqGOLD viral RNA Kit

INTRODUCTION

The peqGOLD viral RNA Kit is designed for isolation of viral RNA from cell free fluids such as plasma, serum, urine and cell culture supernatants.

The kit is also suitable for isolation of total RNA from cultured cells, tissues and bacteria.

RNA purified using the peqGOLD viral RNA Kit method is ready for applications such as RT-PCR

Protocol: Purification of Viral RNA (Spin Protocol)

This protocol is for purification of viral RNA from 140 µl plasma, serum, urine, cell culture.

Important points before starting

■ Read "Important Notes" (pages–30)

■ All centrifugation steps are carried out at room temperature (15–25°C).

Things to do before starting

■ Equilibrate samples to room temperature (15–25°C).

■ Equilibrate Buffer AVE to room temperature for elution in step 11.

■ Check that Buffer AW1 and Buffer AW2 have been prepared according to the instructions on page 32.

■ Add carrier RNA reconstituted in Buffer AVE to Buffer AVL

Important; if not have QIAamp Viral RNA mini , we can use peqGOLD Viral RNA Kit protocoles visit www.peqlab.de

procedure

1. Pipet 560 µl of prepared Buffer AVL containing carrier RNA into a 1.5 ml microcentrifuge tube. If the sample volume is larger than 140 µl, increase the amount of Buffer AVL–carrier RNA proportionally (e.g., a 280 µl sample will require 1120 µl Buffer AVL–carrier RNA) and use a larger tube.

[27] Ready-to-use sensitive substrate for the detection of horseradish peroxidase activity. Absorbs at 450 nm (yellow end-product). Ideal for ELISA and solution assays.

* Fully automatable on the QIAcube. See www.qiagen.com/MyQIAcube for protocols.

2. Add 140 µl plasma, serum, urine, cell-culture supernatant, or cell-free body fluid to the Buffer AVL–carrier RNA in the microcentrifuge tube. Mix by pulse-vortexing for 15 s.

To ensure efficient lysis, it is essential that the sample is mixed thoroughly with Buffer AVL to yield a homogeneous solution. Frozen samples that have only been thawed once can also be used.

3. Incubate at room temperature (15–25°C) for 10 min.

Viral particle lysis is complete after lysis for 10 min at room temperature. Longer incubation times have no effect on the yield or quality of the purified RNA.

Potentially infectious agents and RNases are inactivated in Buffer AVL.

4. Briefly centrifuge the tube to remove drops from the inside of the lid.

5. Add 560 µl of ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the tube to remove drops from inside the lid.

Only ethanol should be used since other alcohols may result in reduced RNA yield and purity. Do not use denatured alcohol, which contains other substances such as methanol or methylethylketone. If the sample volume is greater than 140 µl, increase the amount of ethanol proportionally (e.g., a 280 µl sample will require 1120 µl of ethanol). In order to ensure efficient binding, it is essential that the sample is mixed thoroughly with the ethanol to yield a homogeneous solution.

6. Carefully apply 630 µl of the solution from step 5 to the QIAamp Mini column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini column into a clean 2 ml collection tube, and discard the tube containing the filtrate.

Close each spin column in order to avoid cross-contamination during centrifugation.

Centrifugation is performed at 6000 x g (8000 rpm) in order to limit microcentrifuge noise. Centrifugation at full speed will not affect the yield or purity of the viral RNA. If the solution has not completely passed through the membrane, centrifuge again at a higher speed until all of the solution has passed through.

7. Carefully open the QIAamp Mini column, and repeat step 6.

If the sample volume was greater than 140 µl, repeat this step until all of the lysate has been loaded onto the spin column.

8. Carefully open the QIAamp Mini column, and add 500 µl of Buffer AW1. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.

It is not necessary to increase the volume of Buffer AW1 even if the original sample volume was larger than 140 µl.

9. Carefully open the QIAamp Mini column, and add 500 µl of Buffer AW2. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min. Continue directly with step 11, or to

eliminate any chance of possible Buffer AW2 carryover, perform step 10, and then continue with step 11.

Note: Residual Buffer AW2 in the eluate may cause problems in downstream applications. Some centrifuge rotors may vibrate upon deceleration, resulting in flow-through, containing Buffer AW2, contacting the QIAamp Mini column.

Removing the QIAamp Mini column and collection tube from the rotor may also cause flow-through to come into contact with the QIAamp Mini column. In these cases, the optional step 10 should be performed.

10. Recommended: Place the QIAamp Mini column in a new 2 ml collection tube (not provided), and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.

11. Place the QIAamp Mini column in a clean 1.5 ml microcentrifuge tube (not provided). Discard the old collection tube containing the filtrate. Carefully open the QIAamp Mini column and add 60 µl of Buffer AVE equilibrated to room temperature. Close the cap, and incubate at room temperature for 1 min.

Centrifuge at 6000 x g (8000 rpm) for 1 min.

A single elution with 60 µl of Buffer AVE is sufficient to elute at least 90% of the viral RNA from the QIAamp Mini column. Performing a double elution using 2 x 40 µl of Buffer AVE will increase yield by up to 10%. Elution with volumes of less than 30 µl will lead to reduced yields and will not increase the final concentration of RNA in the eluate.

Viral RNA is stable for up to one year when stored at –20°C or –70°C.

Transformation[28]

Initial cloning should be done in a *recA*– cloning strain, such as NovaBlue, or other similar host that lacks the gene for T7 RNA polymerase. This enables high percentage monomer plasmid yields for examination of the construct sequence, as well as separation of cloning from expression. This separation can be valuable in troubleshooting any difficulties that might arise during later procedures.

The strains described above for cloning and expression with pET vectors can be prepared for transformation by standard procedures. Expect BL21 (an expression strain) and its derivatives to be transformed at about 1/10 the efficiency of the other strains. For convenience and consistent performance, Novagen offers the relevant host strains as prepared competent cells, ready for high-efficiency transformation.

DNA in ligation reactions containing high-quality reagents is suitable for direct addition to Novagen's Competent Cells (no more than 1 µl ligation should be used per 20 µl cells).

Inactivation of the ligase is not required prior to transformation. Plasmid DNA isolated using standard preparation procedures is also usually satisfactory; however, for maximum efficiency, the

[28] Extracted from Novagen

sample DNA should be free of phenol, ethanol, salts, protein and detergents, and dissolved in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) or in water.

Novagen's Competent Cells are provided in 0.2 ml aliquots. The standard transformation reaction requires 20 µl cells, so each tube contains enough cells for 10 transformations. Singles™ Competent Cells are provided in 50 µl aliquots, which are used "as is" for single 50 µl transformations. Note that there are a few steps in the protocol that vary for the Singles™ vs. standard cells. Novagen's NovaBlue and BL21 (DE3) Competent Cells are also offered in a highthroughput 96-well plate format known as HT96™ Competent Cells (see Technical Bulletin 313).

Handling Tips

1. Upon receipt from Novagen, verify that the competent cells are still frozen and that dry ice is still present in the shipping container. Immediately place the competent cells at –70°C or below. For optimal results, do not allow the cells to thaw at any time prior to use.

2. Handle only the very top of the tube and the tube cap to prevent the cells from warming.
Keep the cells on ice whenever possible.

3. To mix cells, flick the tube 1–3 times. *NEVER* vortex the competent cells.

4. To avoid multiple freeze-thaw cycles of the standard 0.2 ml cells, dispense the cells into aliquots after the initial thaw and store them at –70°C or below (note that Singles™ Competent Cells are provided as 50 µl aliquots, which are used "as is" and do not require dispensing. To dispense aliquots of cells from the 0.2 ml stock, remove the stock tube quickly from the ice and flick 1–2 times to mix prior to opening the tube. Remove a 20 µl aliquot from the middle of the cells, and replace the tube immediately on ice. Place the aliquot immediately into the bottom of a pre-chilled 1.5 ml tube, mix by pipetting once up and down, and then immediately close the tube and replace on ice. After all of the aliquots are taken, return any unused tubes to the freezer before proceeding with the transformation.

Plating techniques

1. Remove the plates from the incubator. If plating less than 25 µl of the transformation, we recommend plating onto a pool of SOC, which facilitates even colony distribution on the plate surface. Using a sterile pipet tip, place 40–60 µl of SOC in the center of a plate for a plating cushion.

2. To remove the transformation sample, flick the transformation tube 5–8 times, open the cap and immediately remove the sample volume from the middle of the transformation reaction.

3. Transfer the sample to the plate by dispensing the sample volume into the SOC cushion.
After the sample is out of the pipet tip, use the same tip to pipet up the sample volume's worth of SOC from the cushion edge and dispense that SOC back into the cushion. (This effectively rinses out your pipet tip.)

ColiRollers™ Plating Beads

To use ColiRollers, simply dispense 10–20 beads per plate. The beads can be dispensed before or after pipetting the transformation mix on the plate. Cover the plate with its lid and move the plate back and forth several times. The rolling action of the beads distributes the cells. Several plates can

be stacked up and shaken at one time. After all plates have been spread, discard the ColiRollers by inverting the plate over a collection container. Cover and incubate (step 12 above).
ColiRollers™ Plating Beads are treated glass beads that eliminate the use of the spreader and alcohol flame while evenly and consistently distributing cells without damage.

Standard spreader

Completely immerse the plating spreader (bent glass rod or equivalent) into ethanol and flame to sterilize. After the flame is extinguished, allow the spreader to cool ~10 sec prior to placing the spreader on the plate. Place the spreader on the LB agar at the outside of the plate (not touching the pool of cells). This further cools the spreader on the LB agar before spreading the cells. Slowly turn the plate while supporting the weight of the spreader.
Important: Do not press down on the spreader – use just enough pressure to spread the cells.
Spread until the sample is evenly distributed on the plate. If the plates are fairly dry, the sample and cushion will quickly absorb into the plate. After the moisture is absorbed, do not continue spreading. If the plates are wet, spread until the sample is evenly distributed. Do not spread until the sample and cushion have absorbed completely into the plate, because overspreading can decrease transformation efficiency. Instead, after spreading briefly, allow the plates to sit upright at room temperature for ~15 min prior to placing them in the 37ºC incubator. This will allow excess moisture to absorb into the plates before the plates are inverted and placed in the incubator.
Incubate all plates, cover-side down, in the 37ºC incubator for 15–18 h. To obtain larger colonies, extend the incubation time slightly (1–2 h), but beware of the potential for development of satellite colonies with extended incubations (usually > 36 h at 37°C; satellites are not commonly observed when using carbenicillin or kanamycin). Once the colonies are at the desired size, the plates can be placed at 4ºC.

The Host Strains

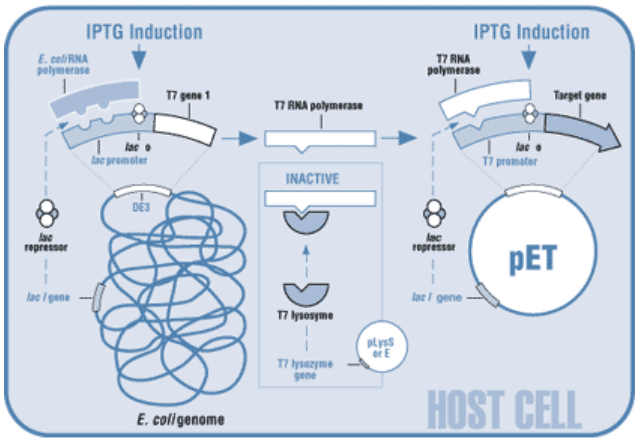
Host Strains[29]

After plasmids are established in a non-expression host, they are most often transformed into a host bearing the T7 RNA polymerase gene (λDE3 lysogen) for expression of target proteins. Figure 1 illustrates in schematic form the host and vector elements available for control of T7 RNA polymerase levels and the subsequent transcription of a target gene in a pET vector. In λDE3 lysogens, the T7 RNA polymerase gene is under the control of the *lacUV5* promoter. This allows some degree of transcription in the uninduced state and in the absence of further controls is suitable for expression of many genes whose products have innocuous effects on host cell growth.

[29] **United States & Canada 800-207-0144**
Germany 0800 6931 000
United Kingdom 0800 622935
Or your local sales office
www.novagen.com

For more stringent control, hosts carrying either pLysS or pLysE are available. The pLys plasmids encode T7 lysozyme, which is a natural inhibitor of T7 RNA polymerase, and thus reduces its ability to transcribe target genes in uninduced cells. pLysS hosts produce low amounts of T7 lysozyme, while pLysE hosts produce much more enzyme and, therefore, represent the most stringent control available in λDE3 lysogens (4).

Figure 1. Control elements of the pET system



Several different host strains are available as λDE3 lysogens. The most widely used host is BL21, which has the advantage of being deficient in both *lon* (5) and *ompT* proteases. Novagen has introduced two derivatives of BL21 designed for special purposes. The B834 series is methionine deficient and, therefore, enables high specific activity labeling of target proteins with ^{35}S-methionine or selenomethionine (6). The BLR strain is a *recA-* derivative that improves plasmid monomer yields and may help stabilize target plasmids containing repetitive sequences. The AD494 strains are thioredoxin reductase (*trxB*) mutants that enable disulfide bond formation in the *E.coli* cytoplasm. This allows for the potential production of properly folded, active proteins (7). Other available strain backgrounds include the K-12 strains HMS174 and NovaBlue, which are *recA-*, like BLR. These strains may stabilize certain target genes whose products may cause the loss of the DE3 prophage. NovaBlue is potentially useful as a stringent host due to the presence of the high affinity *lacI*^{q} repressor encoded by the F episome. In addition, Novagen offers the λDE3 Lysogenization Kit for making new expression hosts with other genetic backgrounds.

2.7 Results / *النتائج*

2.7.1 Amplification of the HLA-A*0201 and β-2m gene from PBMC / مضاعفة جين هلا*0201 وبيتا-2م من المحيط الخارجي لخلايا الدم البيضاء

First: we isolated the total RNA form PBMC as follows:

We drew 10ml of blood from a person, we putted it in 4ml of EDTA (fig.1) to prevent the agglutination (EDTA is an anticoagulant) and we centrifugated it into a centrifugation at 4000rpm for 8 minutes

أولاً: قمنا بعزل الرنا الكامل من المحيط الخارجي لخلايا الدم البيضاء كما يلي:

قمنا بسحب 10مل من دم إنسان، ووضعناه في 4مل من إيثلين ثنائي الأمين رباعي حمض الخل (EDTA) (صورة 1) لمنع تخثر الدم (منع التجلط) ومن ثم نضعها في آلة الطاردة لفصل الدم على

(fig.2), we obtained the result designed in fig.3 then we aspirated the phase 2 that contains the white blood cells with a micropipette 1000µl, we putted it in a 1.5ml microcentrifuge tube finally we isolated the total RNA by the peqGold viral RNA kit (can be stored the result at 4°C).

سرعة 4000 آر.بي.آم لمدة 8 دقائق (صورة 2)، وبعدها نكون قد حصلنا على ثلاث طبقات من الدم كما في الصورة 3 وبواسطة الماصة الأوتوماتيكية الخاصة ب.1000µl نسحب الطبقة الثانية من الدم المحتوية على كريات الدم البيضاء ونضعها في أنبوب 1.5مل ونبدأ بعملية عزل الرنا الكامل بواسطة مجموعة الرنا الفيروسي بك غولد. (تحفظ النتيجة على 4°م)

Figure 1: putting the blood in 4ml EDTA / الصورة 1: وضع 10 مل من الدم في 4مل من مضاد التخثر

Figure 2: centrifugation at 4000rpm for 8min / الصورة 2: عملية الفصل على 4000آر.بي.آم لمدة 8دقائق

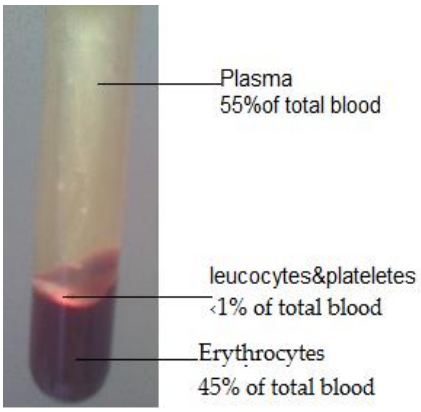


Figure 3: Blood components after centrifugation / الصورة 3: مكونات الدم بعد الفصل.

Second: we retrotranscripted the tRNA to cDNA by M-MLV reverse transcriptase as described in part 3.6 step 3.

Third: we amplified the cDNA by PCR as described in part 3.6 step 4, and we obtained a quantity amplified of HLA-A*0201 and β2-m.

ثانياً: قمنا بعملية إعادة نسخ الرنا الناقل إلى الدنا الناسخ بواسطة أنزيم M-MLV reverse transcriptase كما هو مذكور في الجزء 3.6 المرحلة 3.

ثالثاً: بعدما حصلنا على الدنا الناسخ قمنا بمضاعفة كميته بواسطة آلة تفاعل البلمرة المتسلسل كما هو مذكور في الجزء 3.6 المرحلة 4، وهكذا نكون قد حصلنا على كمية مضاعفة من ال هلا*0201 وبيتا2-آم.

Figure 4: Pipetting of 1µl of M-MLV(RT) / الصورة 4: إمتصاص 1 ميكروليتر من **M-MLV**

Figure 5: PCR machine / **الصورة 5: آلة تفاعل البلمرة المتسلسل**

Fourth: we purified the PCR product on an agarose gel**;**

1. we prepared the agarose gel as follows**:**

The preparation of an agarose gel: - Put 1g of agarose in 100ml of TAE buffer 1×PH and dissolve it at 90°C.

- Let the gel cool down to about 60°C at room temperature.
- Put the gel on the gel rack then insert the comb and let it to solidify.

رابعاً: قمنا بتنقية منتج آلة تفاعل البلمرة المتسلسل بواسطة هلام الأغاروز:

1. قمنا بتحضير الهلام الأعاروزي كما يلي:

طريقة التحضير: – وضعنا 1غ من الأغاروز في 100مل من منظم TAE 1×درجة الحموضة ثم قمنا يتذويبه على 90°م.

- تركناه ليبرد إلى 60°م على درجة حرارة الغرفة.
- أضفناه على طبق الهلام وأضفنا عليه المشط ثم تركناه حتى يجمد.

Figure 6: Dissolve the gel at 90°C with agitation / الصورة 6: تذويب الهلام على 90°م مع التحريك.

Figure 7: putting of the gel on the gel rack / الصورة 7: وضع الهلام على الطبق

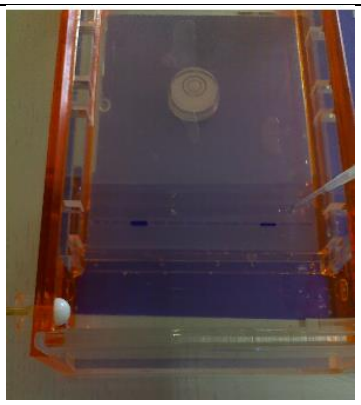


Figure 8: injetion of 10µl of sample into the wells of gel الصورة 8: وضع 10ميكروليتر من العينة / في فتحة الهلام

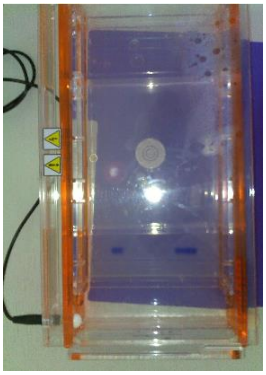


Figure 9: migration of the three bands (2 bands for HLA-A*0201& 1 band for β2-m)

الصورة 9: هجرة البقع الثلاثة (بقعتين للهلا وبقعة لبيتا2–آم)

2. After the solidification of gel, we drew the comb and the border of the gel rack, we added 1liter of TAE buffer to cover all the gel, then we injected 10µl of each sample (we putted in each new eppendorf tube 6 µl of the DNA (HLA-A*0201 or β-2m) + 3µl of glycerol + 3µl of bormophenol blue) into the wells of the gel prepared, we closed the lid of electrophoresis chamber and applied the voltage at 120 volts for 30min, (400mA, 400 Watts).

2. بعد جماد الهلام جيداً، قمنا بسحب المشط وأطراف طبق الهلام، وأضفنا 1 ليتر من منظم TAE 1×درجة الحموضة لتغطية الهلام كاملاً ووضعنا في الفتحات الصغيرة للهلام 10ميكروليتر من كل عينة (تحتوي العينة في كل أنبوب على 6ميكروليتر من الدنا إما الهلا 0201وإما بيتا2–آم + 3 ميكروليتر من الغليسيرول + 3 ميكروليتر من البروموفينول الأزرق) ثم أغلقنا غطاء الرحلان الكهربائي وقمنا بتشغيل التيار الكهربائي على 120فولت لمدة 30 دقيقة.

3. After the migration of bands we turned off the power supply we removed the gel and putted it into a deep vessel we covered it by 250ml of dH_2O and we added 30µl of

3. بعد هجرة بقع الدنا قمنا بإطفاء التيار الكهربائي عن الآلة، أخذنا الهلام ووضعناه في وعاء عميق أضفنا عليها 250مل من الماء المعقم مع 30 ميكروليتر من بروميد الإثيديوم وتركناه لمدة

ethidium bromide and late it for 30min. then we washed the gel 3 times from ethidium bromide with dist.water.(250ml in each times).

WARNING: be curful with the ethidium bromide!!! Use specific gloves, put the waste liquid of ethidium bromide in a specific place, and do not throw it in the nature.
Ethidium bromide could cause a cancer and it is mutagen.

4. We putted the gel on UV-machine to see if we have bands or no, if yes, so the steps are correct and we can continue the protocol.

Result: AlHamdullillah we observed the 3 bands and we could continue the protocol (fig.12).

30 دقيقة، بعدها قمنا بغسله من بروميد الإثيديوم 3 مرات بواسطة الماء المعقم (وضعنا في كل مرة 250مل من الماء المعقم).

تحذير: يجب الحذر في التعامل مع الإتيديوم !!! إستخدم القفازات الخاصة , ضع الماء المحتوية على بروميد الإثيديوم في مكان خاص و لا ترميه في الطبيعة . لأنه قد يسبب السرطان ويحدث الطفرات (التغيير المفاجئ للدنا).

4. بعد غسله جيداً من بروميد الإثيديوم وضعناه على آلة الأشعة ما فوق البنفسجية لنرى إذا كان هناك بقع من الدنا، إذا إن هذه المرحلة ستبين لنا نتيجة ما تم فعله حتى الآن.

النتيجة: الحمدلله تمكنا من رؤية البقع الثلاثة التي تم وضعهم (بقعتين للهلا وبقعة لبيتا2-آم) وتمكنا من تكملة العمل.

Figure 10:injection of 30µl of ethidium bromde in the gel recovered with 250ml dH_2O.

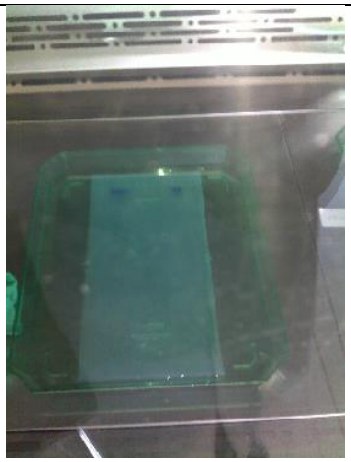


Figure 11: soaking the gel with ethidium bromide for 30min

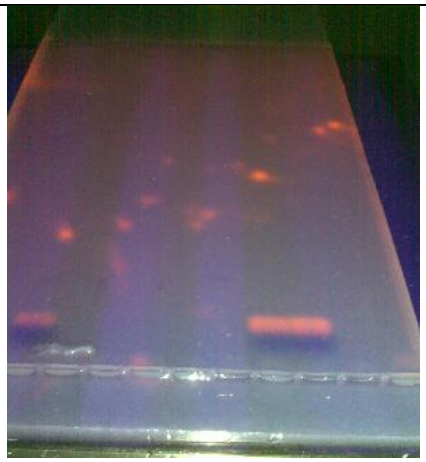


Figure 12: observation of 2 bands of HLA-A*0201 at the right an 1 band of β2-m at the left on UV-machine

5. We marked off the border of each band on the UV-machine (fig.14), then we putted the gel in the deep vessel to cut the bands and should be remove all the excess of gel (fig.15) then we putted each band in a new eppendorf tube (fig.16), we balanced each tube before and after

2. بينما كان الهلام على آلة الأشعة مافوق البنفسجية قمنا بتعيين حدود البقع (الصورة 14)، ثم نقلناه إلى الوعاء العميق ثم قطعنا البقع جيداً مع إزالة الهلام الزائد (الصورة 15) وضعنا كل بقعة في أنبوب خاص (الصورة 16)، ثم قمنا بأخذ

the addition of band in it for obtain the weight of bands to begin the purification of those bands with the Qiaquick gel extraction kit[30] (the weight of HLA-A*0201 was 1.32g, and for β2-m was 1.31g) then we stored the purified DNA at -20°C until use.

وزن كل أنبوب بداخله البقعة من الهلام وأنبوب فارغ لنحصل على الوزن الصافي للهلام لنبدأ بتصفيته عبر مجموعة إستخلاص هلام كياكويك (الوزن الصافي للهلا 0201 كان 1.32غ وبيتا كان 1.31غ) وبعدها قمنا بحفظ الدنا المنقى على – 20°م حتى إستعماله في المراحل القادة.

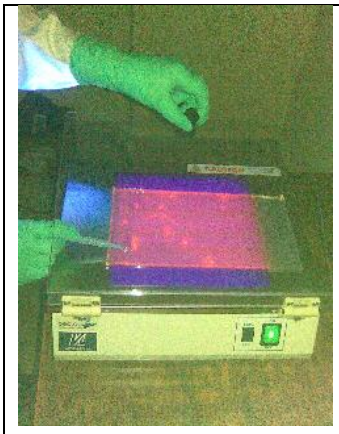


Figure 13: marking off the border of bands

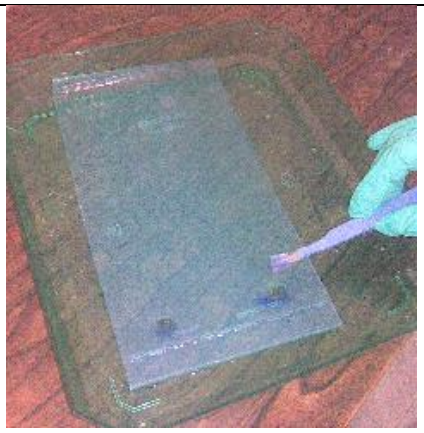


Figure 14: cutting the DNA band from the gel

Figure 15: DNA band in eppendorf tube

2.7.2 Preparation of vector / تحضير الناقل

To digest the vector we assembled the following components in a microcentrifuge tube:

3 µl pET vector

3 µl 10X restriction enzyme buffer

1 µl (10–20 U) EcoR I restriction enzyme

23 µl Nuclease-free water brought to volume

30 µl Total volume

We incubated at the appropriate temperature (usually 37°C) for 2–4 h in the water bath. We ran a 3 µl sample on an agarose gel to check the extent of digestion (fig.16), when digestion is complete, we added 1µl of calf intestinal alkaline phosphatase (we diluted 0.2µl of calf intestinal alkaline phosphatase in 0.8 µl of water for obtain 0.05U) directly to the remainder of the digestion. We incubated at 37°C for 30 min in the water bath then we injected the sample in 4

لقطع الناقل قمنا بجمع المكونات التالية في أنبوب النابذة:

3 ميكروليتر من الناقل pET.

3 ميكروليتر من منظم أنزيم القطع 10×.

1 ميكروليتر (10–20وحدة) من أنزيم القطع إيكو.آر.وان

23 ميكروليتر من ماء خالٍ من النيوكياز.

الحجم النهائي في الأنبوب 30 ميكروليتر.

قمنا بحضن الأنبوب لمدة ساعتين على 37°م في حوض من الماء. ومن ثم وضعنا 3 ميكروليتر من العينة على هلام الأغاروز لنتحقق من قطعه (صورة 16)، وعندما تأكدنا من تقطيعه أضفنا 1 ميكروليتر من أنزيم الفوسفاتيز القلوي المعوي للعجل (ولكن لنحصل على تركيز 0.05وحدة من الأنزيم أضفنا 0.8ميكروليتر من الماء على 0.2ميكروليتر من الأنزيم) مباشرة لإبقاء عملية

[30] For more detail see MEGBI training courses book part I page 54-58.

wells in the gel (fig.17), we ran the gel to separate the linear plasmid from nicked and supercoiled species. We visualized the DNA band with a long wave UV light source (fig.18) and we cut the band from the gel using a clean razor blade.

WARNING: Avoid over exposure to the light source, which can cause nicks and double strand breaks in the DNA.

We balanced the bands to begin that purification with QIA quick gel extraction kit[31] Resuspended the final product in a total volume of 30 µl (usually about 50 ng/µl DNA). Assume recoveries in the range of 50% for the ligation step. Then we stored the treated vector at –20°C until use.

القطع ووضعناه في حوض ماء على 37°م لمدة 30دقيقة ثم قمنا بوضعه في 4 فتحات من الهلام الأغاروزي لتنقيته (الصورة 17)، بعد هجرة البقع على الهلام كما في المرحلة السابقة قمنا بتقطيعهم من الهلام الزائد بواسطة مشرط حاد ونظيف (الصورة 18).

تحذير: يجب تجنب تعريض الزائد للهلام على الأشعة ما فوق البنفسجية لأن ذلك قد يسبب شقوق وكسر للحبلين المزدوجين للدنا.

ومن ثم قمنا بقياس وزن البقع لنبدأ بعملية تنقيتهم من الهلام بواسطة مجموعة إستخلاص هلام كياكويك. الحجم النهائي من المنتج هو 30ميكروليتر ويمكن حفظه على –20°م إلى حين إستعماله مرة أخرى.

Figure 16: visualization the digestiof vector in the gel

Figure 17: injection the sample in 4 wells in gel

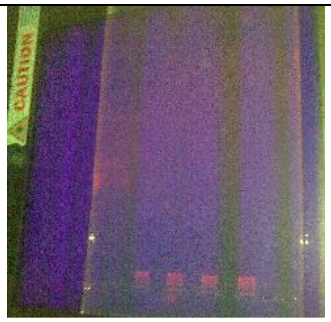


Figure 18: observation the bands in UV-machine

2.7.3 Ligation / عملية الربط

We prepared 3 eppendorfs tubes and we marked each eppendorf (the first for the ligation of vector with HLA-A*0201, the second for the vector with β2-m, the third for the vector with T7 RNA Polymerase).

Figure 19: Marking of each tube

في هذه العملية قمنا بتجهيز 3 أنابيب من النابذة مع الكتابة على كل أنبوب ما الدنا الذي سيحتويه (مثلاً: الأنبوب الأول كتبنا عليه الناقل مع الهلا0201، الثاني الناقل مع بينا–2آم أما الثالث كتبنا عليه الناقل مع تي7 بوليميراز الرنا.)

[31] Refer to MEGBI training courses book part I page 54-58.

We added in each eppendorf tube (1.5ml):
2 µl 10X Ligase Buffer (200 mM Tris-HCl pH 7.6,
8 µl 25 mM MgCl2
2 µl 100 mM DTT
1 µl 10 mM ATP
2 µl 50 ng/µl prepared pET vector
1 µl T4 DNA ligase, diluted (with ligase dilution buffer) 0.2–0.4 Weiss units/µl
2 µl Prepared target gene insert (0.2 pmol)
2 µl Nuclease-free water to volume
20 µl Total volume
Then gently we mixed by stirring with a pipet tip, we incubated at 16°C to overnight.

ثم أضفنا في كل أنبوب (1.5مل):

2 ميكروليتر من منظم الليغاز 10×.

8 ميكروليتر من كلور الماغنيزيوم 25mM.

2 ميكروليتر من 100mM DTT.

1 ميكروليتر من 10Mm ATP.

2 ميكروليتر من الناقل pET المحضر 50ng/µl.

1 ميكروليتر من ليغاز الدنا تي4، المخفف (مع المنظم المخفف ليغاز) 0.2–0.4 وحدة/ ميكروليتر.

2 ميكروليتر من الدنا المحضر (0.2 pmol).

2 ميكروليتر من ماء خالٍ من النيوكلياز.

20 ميكروليتر الحجم النهائي.

ومن ثم مزجناهم بلطف بواسطة الممصة صعوداً ونزولاً وتركناهم على 16°م طيلة الليل.

2.7.4 Transformation / النقل عملية

Transformation into host strain for cloning

In this step we would to prove if the ligation of the insert with the vector without adapter was possible or no, for this reason we transformed them in the host strain for cloning then we platted them and if in the next day the colonies was amplified on the plate this mean that ligation is possible and vice versa.

For this step we brought a plate contains e.coli from the hospital because when we worked in the e.coli that presented in our lab we found it death and not lost the time we worked this step as follows:

1- We took some colonies from the plate then we putted it in 5ml LB medium.

2- We incubated over night at 37°C with shaking

3- In the second day we observed a big quantity

عملية النقل إلى داخل خلايا الإستنساخ

في هذه الدرحلة أردنا أن نقوم بتجربة ربط الدنا الزائد بالناقل من غير الدوصل) adapter (، وللتأكد من ذلك يجب نقل الدنتج من عملية

الربط إلى داخل خلايا البكتًيا وزرعها على وسط غذائي ملائم ثم مراقبتهم في اليوم التالي فإذا تبين أن خلايا البكتيريا تكاثرت فهذا يعني أن عملية الربط قد لصحت والعكس صحيح.

ولذذه التجربة جلبنا بكتًيا مزروعة من الدستشفى وذلك لأن البكتًيا التي كانت موجودة عندنا في الدختبر وجدناه ميتة بينما كنا لصهزبا لنعمل بها ولكي لا نخسر الكثير من الوقت بإنتظار إحضار بكتيريا أخرى من الشركة، قمنا بالعمل ببكيريا الدستشفى كما يلي:

1 –أخذنا بعض من البكتًيا الدزروعة في صحن بتًي ووضعنام في 5 مل من وسط آل بي.

2 –مع الإنتزاز ° .حضنام طيلة الليل على 37 م

3 –في اليوم التالي لاحظنا أن خلايا تكاثرت بشكل ملحوظ في الوسط آل بي عندئذ وزعنا كمية الوسط التي كانت في الأنبوب إلى 4أنابيب نابذة ووضعنام في الثلج لددة 10 دقائق، ومن ثم

of e.coli in the medium we distributed the medium in 4 eppendorf tubes and we chilled them on ice for 10 min, centrifuged them at 4000 rpm for 10 min at 4°C, discarded the supernatant and resuspended the pellets each in 600 µl of cold 0.1M calcium chloride, leaved them on ice for 25 min, centrifuged them at 4000 rpm for 10 min at 4°C, resuspended each pellet in 60 µl of cold 0.1M calcium chloride (this step for competent cells) then we let some microliter for the next step and we stored the remaining at -20°C

4- We did the transformation as follows:

We pipetted 20 µl aliquots of cells e.coli from the hospital into pre-chilled tube we added 1 µl of each ligation reaction (we added the pET that without cutting as control positive), stirred gently to mix and we returned to the ice we incubated 5 min on ice , we placed the tubes in a 42°C water for exactly 30 sec. (do not shake), we placed the tubes on ice for 2 min, we added 80 µl LB medium to each tube, kept the tube on ice until all have received LB, incubated for 1 hour at 37°C with shaking.

5- We Platted them onto plate contain LB medium with ampicillin then incubated overnight at 37°C

6- We observed in the second day colonies on the pate and that mean the ligation is possible and we can continue with the expression.

7- For the plate that contains the pET (the control positive) we took some colonies from it and we putted it the 2.5ml LB medium with ampicillin then we incubated it overnight at 37°C for 12-16 hours.

8- In the next day we centrifuged it and we purified it with Qiaprep spin Miniprep kit then we stored the result at -20°C.

ثم إرمي ، °نبذنام على 4000 آر.بي.آم لددة 10 دقائق على 4م

السائل وأضف 600 ميكروليتً من كلورايد الكالسيوم 0.1M البارد على الدتًقد في الأنبوب وإتركو في الثلج لددة 25 دقيقة ومن ثم ° بعدنا إنبذنم على 4000 آر.بي.آم لددة 10 دقائق على4 م أضف 60 ميكروليتً من كلورايد الكالسيوم 0.1M على الدتًقد في

الأنبوب(نذه الدرحلة بي لفتح الخلايا)وأخيراً تركنا بعض الديكروليتً لنعمل بهم في الدرحلة التالية وقمنا بتخزين الباقي في °.الثلاجة على– 20م

4– قمنا بعملية النقل كالتالي :أخذنا 20 ميكروليتً من خلايا البكتًيا ووضعنام في أنبوب بارد وأضفنا 1 ميكروليتً من كل من تفاعل الربط(وأضفنا أيضاً إحدى الأنابيب الناقل pET لوحده كتحكم إيجابي)، حركنام بنعومة لخلطهم ووضعنام في الثلج لددة لددة 30 ثانية(من دون 5 ◦ دقائق ثم في الداء الساخن على 42 م

برريك)ثم في الثلج مرة ثانية لددة 2 دقيقتين وأضفنا 80 ميكروليتً من وسط آل بي لكل أنبوب، وتركنام في الثلج إلى أن يصل الوسط إلى الجميع في الأنبوب ثم وضعنام في الحاضنة لددة ساعة مع التحريك ◦ .على 37 م

5 –ثم زرعنام على صحن بتًي المحتوي على وسط آل بي مع ◦.الأمبيسيلين ومن بعدنا وتركنام طيلة الليل على 37 م

6 –وفي اليوم الثاني لاحظنا لرموعات متكاثرة من البكتًيا على الصحن وذا يعني أن عملية الربط لشكنة ويدكننا تكملة العمل في التعبير عن البروتين.

7 –أما بالنسبة لصحن الناقل الذي أخذناه كتحكم إيجابي أخذنا منو بعض لرموعات البكتًيا ووضعنام في 2.5 مل من لددة ◦ وسط آل بي مع الأمبيسيلين وتركنام طيلة الليل على 37 م 12–16ساعة.

8 –وفي اليوم التالي نبذنام ثم قمنا بتصفيتهم بواسطة لرموعة "كيابرب سبين مينيبرب "والناتج الذي حصلنا عليو خزناه على– 20 ◦م.

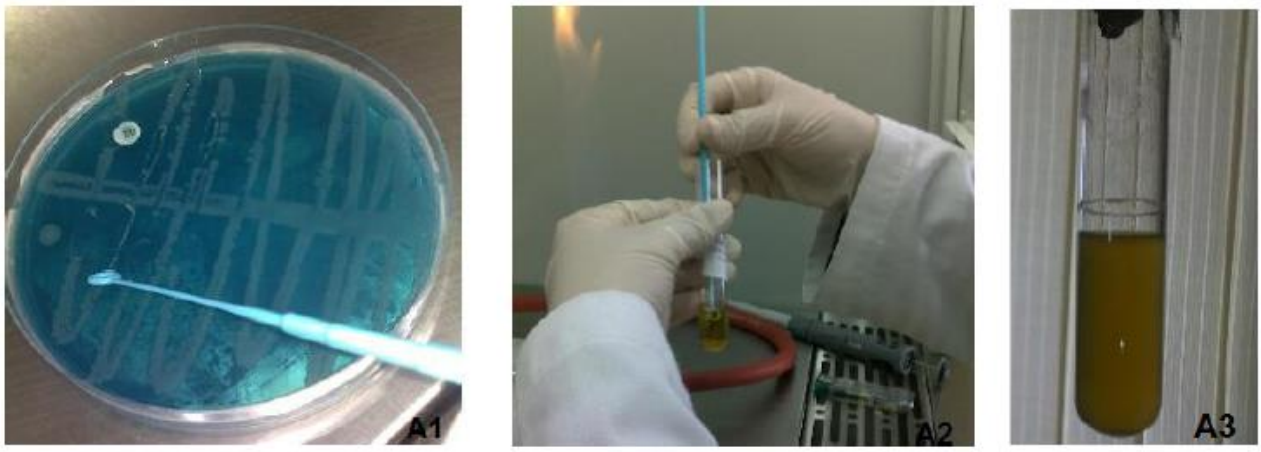


Figure 20: A1) take some colonies from the plate. A2) put the colonies in LB medium and let overnight at 37°C with shaking, next day observe the result as in A3, continue with the competent cells, and the transformation.

الاصورة : 20 أ)خذ بعض مجموعات البكتريا من الاحن، أ)2 وضعهم في وسط LB وتركهم طيلة الليل على 37 م °مع التحريك، في اليوم التالي لاحظنا أن الوسط تغير لونو كما في أ3 ، أكمل في فتح خلايا البكتربا، ومن ثم في عملية النقل

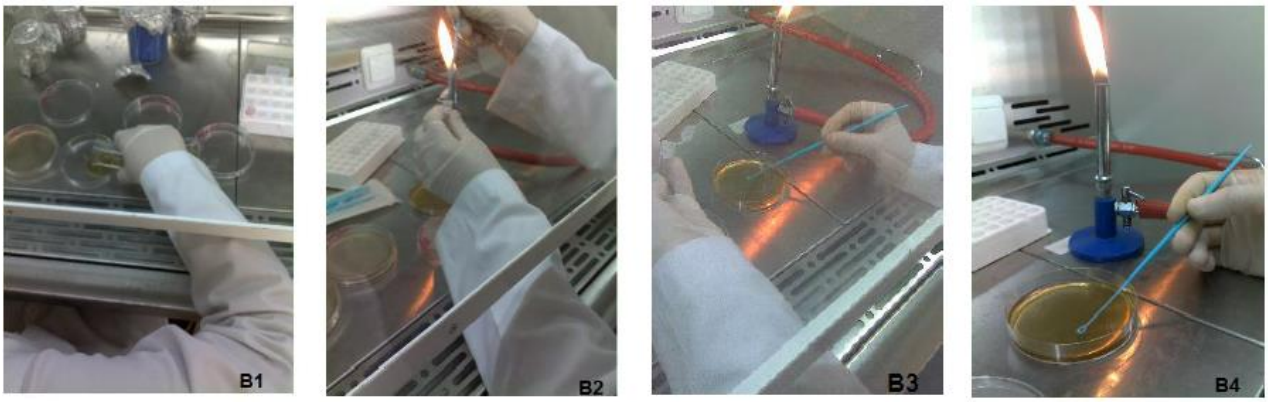


Figure 21: after preparing the LB medium with the ampicillin, B0) put it in the plate with attention from introducing air bubbles, B0) take some microliter from the transformation and plate it on the medium as fig. B3, B4)

الاصورة : 21 بعد بحضير وسط آل بي مع الآمبيسلين، ب)2 ضعه في الاحن مع الإنتباه من تكوين فقاقيع من الهواء، ب)2 خذ بعض الميكروليتر من عينة الناقل وازرعهم على الوسط (.كما في ب 3 و ب4

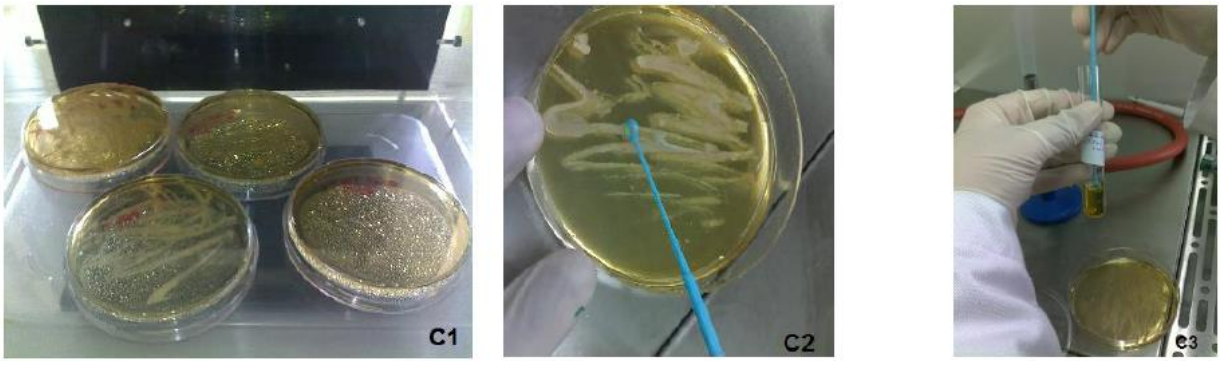


Figure 22: C1) after plating, incubate the plates overnight at 37°C, C2) in the next day take some colonies from the plate and put it in LB medium with ampicillin as C3) and let it between 12-16h at 37°C.

الصورة 22: ج 1) بعد عملية الزرع، أترك الصحون طيلة الليل على 37م°، ج2) في اليوم التالي خذ بعض المجموعات من الصحن وضعها في وسط آل بي مع أمبيسيلين كما في ج3) وأتركهم ما بين 12– 16 ساعة على 37م°.

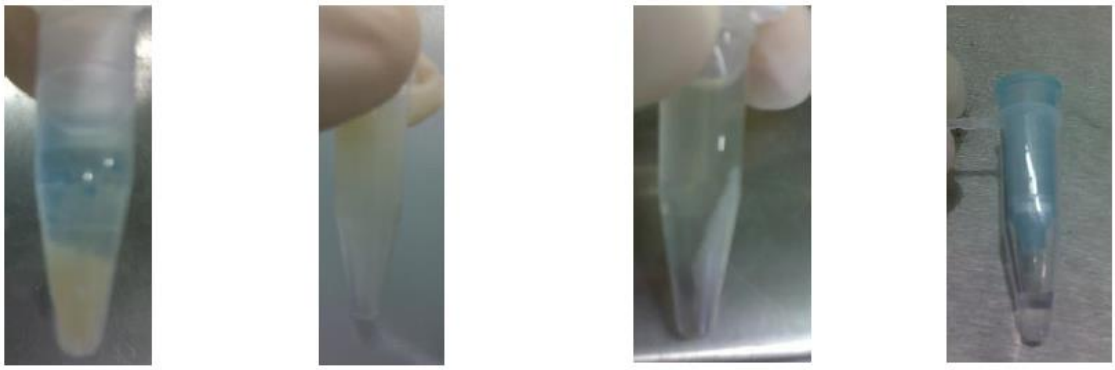


Figure 23: this the result in each step when worked in the purification of plasmid not cutting for restored at -20°C./ .20م°– الصورة 23: هذه الأنابيب هي نتيج كل مرحلة من مرحلة تنقية الناقل الغير مقطع والذي سيحفظ على

عملية النقل إلى داخل الخلايا المعبرة

في هذه المرحلة قمنا بتجربة العمل على الخلايا البكتيرية المخصصة للإستنساخ مع إضافة جين بوليمراز الرنا ت7 المؤتلف مع الناقل pET (الذي جهز في مرحلة الربط) وأضفناه مع كل عملية نقل سواء كانت نقل هلا 0201 المؤتلف أو بيتا2–آم المؤتلف ثم زرعناهم على صحون بتري وأكملنا بهم العمل حتى فحص إيليزا لنرى إن كان هناك مركب فهذا يعني أن هذه المرحلة صحيحة والعكس صحيح.

Transformation into expression host

We tried in this step to work in host for cloning and we added in it the T7 RNA Polymerase recombinant with the pET vector (step of ligation) and we added it with the transformation of HLA-A*0201 recombinant and β0-m recombinant then we platted them onto petri dishes and we continued the protocol until ELISA test for see if there was a complex that meaning there is a protein and this step is correct and vice versa.

To do this first should be prepared the host strain, second should be done the transformation, the plating then the extraction the protein from the strain and purified it, finally should be done the ELISA test.

We took the E.coli top 10 from -70°C and that should be competent and we worked in it but when we plated onto petri dish we didn't saw in the second day any colonies, for this reason we think that the e.coli was died but for prove that

we putted some micro liter of this e.coli into 5ml LB medium and we incubated them at 37°C with shaking for overnight and in the second day we saw the amplification of e.coli and that meaning the e.coli wasn't died but it wasn't competent, we did the competent for the e.coli as follows:

We dispensed the 5ml of sample that in the tube in 4 eppendorf tube 1.5ml and we let them in glace for 10 min, then we centrifuged them at 4000 rpm at 4°C for 10 min, we discarded the supernatant and we added 600µl of CaCl2 0.1M cold, we let them in glace for 25 min then we centrifuged at 4000 rpm for 10 min at 4°C finally we resuspended each pellet in 60µl of CaCl2 0.1M cold, then we stored 3 eppendorf tubes at -20°C and we let one eppendorf for use it in the transformation.

Secondly, we did the transformation as follows:

We pipetted 20 µl aliquots of cells top 10 into pre-chilled tube we added 1 µl of each ligation reaction (first tube contains ligation of HLA-A0201 and T7RNA polymerase, second it contains the ligation of β0-m and T7RNA polymerase), stirred gently to mix and we returned to the ice, incubated 5 min on ice, placed the tubes in a 42°C water for exactly 30 sec. (do not shake), turned the tubes on ice for 2 min, added 80 µl LB medium to each tube kept the tube on ice until all have received LB, incubated for 1 hour at 37°C with shaking.

then we platted them onto 2 plates contain LB medium with ampicillin then

ولفعل ذلك يجب أولاً تحضير سلسلة الخلايا، ثانياً يجب نقل المنتج من الربط إلى الخلايا المحضرة ومن ثم زرعهم ومن بعدها إستخراج التعبير البروتيني من سلسلة الخلايا وتصفيته، وأخيراً يجب التأكد منه بواسطة إيليزا.

في البداية أخذنا بكتريا إشيريكي القلونية top 10 من درجة –70م° حيث يجب أن تكون مفتوحة إلا أن أثناء العمل وزرعها على صحن بتري لم يتبين لنا في اليوم التالي أنها تكاثرت لهذا السبب ظننا بأنها ميتة وللتاكد من ذلك قمنا بوضع بعض الميكروليترات في 5 من وسط آل بي وتركناهم طيلة الليل على 37م° مع التحريك وفي اليوم الثاني لاحظنا أن البكتريا تكاثرت وهذا يوكد بأن البكتريا ليست ميتة ولكن كانت غير مفتوحة لذا قمنا بعملية فتحها على الشكل التالي:

وزعنا ال5مل من وسط آل بي المتكاثر فيها البكتريا على 4 أنابيب نابذة (1.5مل) وتركناهم في الثلج لمدة 10 دقائق، ثم نبذناهم على 4000آر.بي.آم لمدة 10 دقائق على 4م°، ومن بعدها رمينا السائل الطائف في الأنبوب ووضعنا 600 ميكروليتر من كلورايد الكالسيوم 0.1M البارد، ثم تركناهم في الثلج لمدة 25 دقيقة، ثم نبذناهم على 4000آر.بي.آم لمدة 10 دقائق على 4م°، وأخيراً رمينا السائل الطائف في الأنبوب ووضعنا 60 ميكروليتر من كلورايد الكالسيوم 0.1M البارد، ثم قمنا بتخزين 3 أنابيب على –20م° وتركنا أنبوب لنكمل العمل به في عملية النقل.

ثانياً قمنا بعملية النقل كالتالي:

أخذنا 20 ميكروليتر من خلايا top 10 ووضعناهم في أنبوب مبرد وأضفنا 1 ميكروليتر من تفاعل الربط (الأنبوب الأول يحتوي على رابط هلا0201 مع رابط بوليمراز الرنا ت7، الثاني يحتوي على رابط بيتا2–آم مع رابط بوليمراز الرنا ت7)، حركناهم بنعومة لخلطهم وأعدناهم إلى الثلج لمدة 5 دقائق ثم وضعناهم في الماء الساخن على 42م° لمدة 30 ثانية (من دون تحريك) ثم في الثلج مرة ثانية لمدة 2 دقيقتين وأضفنا 80 ميكروليتر من وسط آل بي

incubated overnight at 37°C, we observed in the second day colonies on the plate, we took some colonies from each tube and putted in 2.5ml LB medium contains ampicillin we incubated them for 12-16 hours at 37°C then we centrifuged them at 4000rpm for 5 min, resupended the pellets in 229.84 µl of tris HCl (10mM) and we added in it 25 µg lyososyme, 10 µl phenylmethylsulfonylfluoride (50mg/ml), 0.08 µl DNase (1000U) 0.08 µl RNase (10mg/ml) 1 µl EDTA (1mM), incubated at 22°C for20 min, heat shock for 40s in a boiling water, centrifuged at 10000×g for 20min, washed the pellet with 250 µl 10 mM tris HCl, PH 8, dissolved it in 125 µl of 100mM tris HCl, PH8, 8M urea, then should be centrifuged at 4°C for 1 hour at 150000×g but because we didn't have a centrifuge it speed more than 13000×g we centrifuge them at 13000×g for 2 hours and saw a pellet for this reason we continued in this way,

لكل أنبوب، وتركناهم في الثلج إلى أن يصل الوسط إلى الجميع في الأنبوب ثم وضعناهم في الحاضنة لمدة ساعة على 37 م° مع التحريك.

ثم زرعناهم على صحنين بتري المحتوي على وسط آل بي مع الأمبيسيلين ومن بعدها تركناهم طيلة الليل على 37 م°، وفي اليوم التالي لاحظنا تكاثر البكتريا على الوسط، فأخذنا بعض من المجموعات المتكاثرة ووضعناهم في 2.5 مل من وسط آل بي مع الأمبيسيلين وتركناهم على 37 م° لمدة 12–16ساعة ومن ثم نبذناهم على 4000 آر.بي.آم لمدة 5 دقائق، ومن أضفنا على المترقد في أسفل الأنبوب 229.84 ميكروليتر من تريس حمض الهيدروكلوريك (0.1M) وأضفنا عليه 25 ميكروغرام من اللايزوزيم، 10 ميكروليتر من فنيل ميثيل سولفونيل الفلورايد (50mg/ml)، 0.08 ميكروليتر من أنزيم الدنا (1000U) و 0.08 ميكروليتر من أنزيم الرنا (10mg/ml)، 1 ميكروليتر من إي.دي.تي.آي (1mM)، ثم تركناهم على 22م° لمدة 20 دقيقة، ومن بعدها قمنا بصدمة حرارية للأنبوب لدة 40 ثانية في ماء مغلية، ثم نبذناهم على 10000×g لمدة 20 دقيقة، ومن ثم قمنا بغسل المترقد في أسفل الأنبوب ب250 ميكروليتر من تريس حمض الهيدروكلوريك (10mM)، درجة الحموضة 8، ومن ثم ذوبه في 125 ميكروليتر من تريس حمض الهيدروكلوريك (100mM)، درجة الحموضة 8، مع اليوريا (8M)، ثم كان يجب نبذهم على 4م° لمدة ساعة على 150000×g ولكن بما أن النابذة الموجودة في المختبر السرعة القصوى لديها 13000×g قمنا بنذ الأنبوبين على 13000×g لمدة 2ساعتين وعندما لاحظنا أن هناك كمية من المترقد في أسفل الأنبوب أكملنا العمل على هذا النحو،

Figure 24: after incubation the colonies that contain the insert recombinant,D1) centrifuge them at 4000rpm for 5 min and obtain the result as D2), then resuspend the pellet with the buffer as D3) and incubated at 22°C for 20 min as D4) then transfer them to microcentrifuge tube, heat shock 40s as D5).

الصورة 24: بعد حضن المجموعات التي تحتوي على الدنا في الوسط، د1) انبذ الوسط على 4000أر.بي.آم لمدة 5 دقائق وبعدها ستحصل على النتيجة كما في الصورة د2)، ومن بعدها ذوب المترقد بالمنظم كما في د3) واحضنه على 22م° لمدة 20 دقيقة كما في د4) وانقلهم إلى أنابيب نابذة وقم بصدمة حرارية لهم على 40 ثانية كما في د5).

We purified the protein of HLA-A2020 and β0-m by anion exchange chromatography Q sepharose fast flow (QFF) as follows:

1. We filled the syringe with the start buffer, removed the stopper and connected the column to the syringe with the provided connector, drop to drop to avoid introducing air into the column.

2. Removed the snap-off end at the column outlet.

3. Washed out the preservatives with 5 column volumes of start buffer, at 1ml/min for the HiTrap 1ml.

4. Washed with 5 column volumes of elution buffer.

5. Finally equilibrated with 5 column volumes of start buffer.

6. applied the sample (the protein of HLA-A0201) at 1ml/min for HiTrap 1ml using a syringe fitted to the luer connector

7. Washed with at least 5 column volumes of start buffer or until no

قمنا بتصفية بروتيين الهلا0201 وبيتا2-آم بواسطة غروماتوغرافيا تبادل الإيون السيفاروزي للتدفق السريع كما يلي:

1. أملأنا الإبرة بمنظم البدء، ثم أزلنا السدادة التي على العامود وأوصلنا العامود بالإبرة بالرابط المناسب، وبحذر ولتجنب دخول الهواء على العامود وضعنا نقطة نقطة من الإبرة على العامود إلى أن يلصق به.

2. أزلنا النهاية المضافة عند أسفل العامود.

3. قمنا بغسل المواد في العامود ب 5 مرات من حجم العامود بمنظم البدء، على 1مل/بالدقيقة لكل 1مل من عامود "هاي ترب".

4. قمنا بغسل ب 5 مرات من حجم العامود بمنظم الإستخراج.

5. أخيراً قمنا بتعديل العامود ب 5 مرات من حجم العامود بمنظم البدء.

6. وضعنا العينة من البروتين المراد نتقيته (الهلا0201)على 1مل/بالدقيقة لكل 1مل من عامود "هاي ترب" بإستخدام الإبرة الموصلة الرابط المناسب.

material appears in the effluent.

8. Eluted with 5-10 column volumes of elution buffer.

9. After completed elution, regenerated the column by washing with 5 column volumes of regeneration buffer (elution buffer) followed by 5-10 column volumes of start buffer. The column is now ready for a new sample.

10. Applied the sample (the protein of β0-m) at 1ml/min for HiTrap 1ml using a syringe fitted to the luer connector.

11. Washed with at least 5 column volumes of start buffer or until no material appears in the effluent.

12. Eluted with 5-10 column volumes of elution buffer.

13. When we finished the purification of the entire sample we rinse the column with water then washed with 5 column volumes 20 % ethanol at 1ml/min for the HiTrap 1ml to prevent microbial growth. Sealed the column with the supplied stoppers. And stored at 4°C to 30°C.

7. قمنا بغسل العامود ب 5 مرات من حجم العامود بمنظم البدء.

8. ثم إستخرجنا العينة ب5 مرات من حجم العامود بمنظم الإستخراج.

9. بعد إكمال عملية الإستخراج، قم بغسل العامود ب 5 مرات من حجم العامود بمنظم الإستخراج ثم أيضاً ب 5 مرات من حجم العامود بمنظم البدء. والآن أصبح العامود جاهزاً لتنقية عينة جديدة.

10. أضفنا العينة الثانية المراد تنقيتها (البيتا2-آم) على 1مل/بالدقيقة لكل 1مل من "هاي تراب" بإستخدام الإبرة الموصولة بالرابط المناسب.

11. قمنا بغسل العامود ب5 مرات من حجمه بمنظم البدء.

12. قمنا بعملية الإستخراج بإضافة 5 مرات من حجم العامود من منظم الإستخراج.

13. عند الإنتهاء من نتقية العينات قمنا بغسل العامود بالماء ومن ثم بالأيثانول 20% على 1مل /بالدقيقة لكل 1مل من "هاي تراب" لمنع نمو الجراثيم.

وأخيراً قمنا بختم العامود بالسدادات المزودة والمناسبة. ووضعنا العامود على درجة حرارة بين 4م° و 30م°.

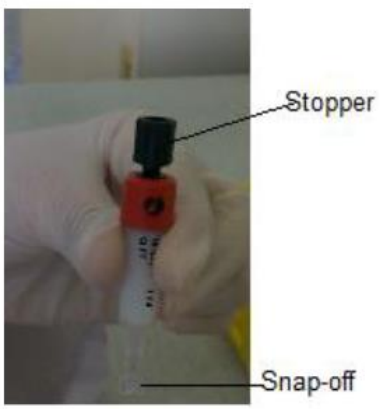


Figure 25: E1) prepare the syringe with the provider connector and fill it with the start buffer as E2), remove the stopper from the column and connect it with the syringe by drop drop as E4) to avoid introducing air to the column.

الصورة 25: هـ1) جهزّ الإبرة بالرابط المناسب ومن ثم قم بملئها بمنظم البدء كما في هـ2)، أزل السدادة من أعلى العامود وقبل بإيصال الإبرة بالعامود قم بوضع بعض النقاط على العامود لمنع دخول الهواء كما في هـ4).

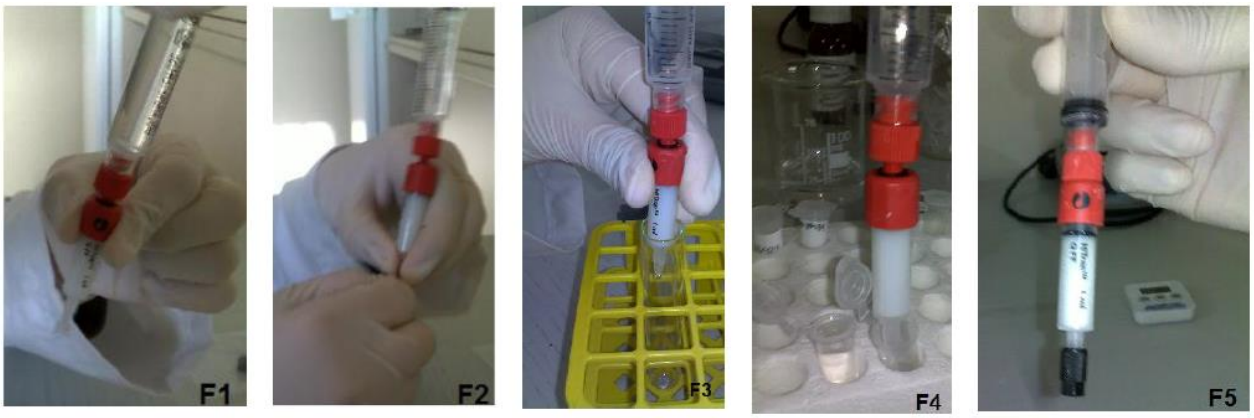


Figure 26: after the connection of syringe with the column remove the snap-off end the column as F2) wash the column with the start buffer and the elution buffer as F3) then elute the sample in a sterile eppendorf tube as F4) then when it finish the rinsing of column seal it with the supplied stopper.

الصورة 26: بعد ربط الإبرة بالعامود قم بإزالة النهاية المضافة للعامود كما في و2) ثم قم بغسل العامود بمنظم البدء ثم بمنظم الإستخراج كما في و3) ثم قم بإستخراج العينة وضعها في أنبوب نابذة كما في و4) وعند الإنتهاء من إستخراج العينة قم بغسل العامود وتنظيفه ثم أغلق الفتحات بالسدادات المناسبة.

Finally we began in the ELISA test as follows:

In the first day we dilute 5 µl of pan specific mouse anti HLA class I antibody, W6/32 in 1ml of carbonate buffer (0.1M) PH 9.6, and we putted 50 µl/well in A1, A2, A3, A4, A5, C1, C2, C3, C4 and we let it at 4°C for overnight.

In the second day we added 350 µl/well 10% w/v protein block to block the residual bind, then we washed twice with 600 µl/well of 0.05% tween in PBS at room temperature to remove the unbound W6/32 and blocking reagent, then we diluted the purified recombinant HLA-A0201 molecule 100 fold into 100µl 0.3 mM tris maleat buffer, PH 6.6, containing human β0-m (100nM), peptide(optimal 1nM to 1µM) and lutrol F-68 (1g/L) and we

وأخيراً بدأنا في فحص إيليزا كما يلي:

في اليوم الأول قمنا بتخفيف 5 ميكروليتر من pan specific mouse anti HLA class I antibody, W6/32 في 1مل من منظم الكاربونات (0.1M) درجة الحموضة 9.6 ، ووضعنا 50 ميكروليتر/في كل من الفتحات التالية: أ1، أ2، أ3، أ4، أ5، ج1، ج2، ج3، ج4 وتركناهم على 4م° طيلة الليل.

وفي اليوم الثاني أضفنا 350 ميكروليتر/بالفتحة من 10% من البروتيين المانع لمنع أي ربط آخر، ومن بعدها قمنا بغسل الفتحات مرتين ب600 ميكروليتر/بالفتحة من 0.05% من التويين في PBS على درجة حرارة الغرفة لإزالة الW6/32 الغير مربتط بالفتحة وإيقاف ما تب

incubated them at 18°C for 48 hours.

In the fourth day of ELISA test we diluted 2 µl fo the sample reaction in 10 µl of protein block with 40 µl PBS 0.05% Tween then we added 50 µl of the sample in each this well C1, C2, C3, C4 and we added on A1,A2, A3, A4, A5, the same sample but without dilution and we let the plate for 2h at 4°C, then we washed 6×times 300µl/well with PBS 0.05% Tween at room temperature, and for detecting the binding complex, we incubated the plate for 1h at 4°C, with 49 µl of post primary block + 1 µl of protein block, then we washed 6×times 300µl/well with PBS 0.05% Tween, we added one drop of Novolink polymer /well and incubated them for 30min at room temperature to enhance the detection, then we washed 6×times 300µl/well with PBS 0.05% Tween, we added 3 µl of 3,3′5, 5′-tetramethylbenzidine hydrogenperoxide in A1, A2, A3, C1, C2, and we added 3 µl of DAB Chromogen in A4, A5, C3, C4, and we observed directly a change of color in A1, A2, A3, C1, C2 from incolor to blue color and after 30 min we observed that color was change to yellow and that was meaning there is a complex and AlHamdullillah we successful in the protocol and in our experiment.

قي من الكواشف، ثم قمنا بتخفيف جزيئات الهلا المؤتلف المنقى في 100 ميكروليتر من منظم tris maleat buffer (0.3mM) درجة الحموضة 6.6، ويحتوي على البيتا2-آم (100nM)، البيتيد (1nM to 1µM) واللوترول ف-68 (1g/l) وتركناهم لمدة ساعة على 18م° لمدة 48 ساعة.

وفي اليوم الرابع من فحص إيليزا قمنا بتخفيف 2 ميكروليتر من تفاعل العينة في 10 ميكروليتر من البروتيين المانع مع 40 ميكروليتر من PBS مع0.05% من التويين، ثم أضفنا 50 ميكروليتر من العينة في كل من الفتحات التالية ج1، ج2، ج3، ج4 وأضفنا في الفتحات أ1، أ2، أ3، أ4، أ5 نفس محتويات العينة ولكن من غير تخفيفها ثم تركناهم على 4م° لمدة ساعتين، وبعدها قمنا بغسل الفتحات 6 مرات ب300ميكروليتر من PBS مع التووين على درجة حرارة الغرفة، ولإستبان المركب المؤلف من الهلا والبتيد أضفنا 49 ميكروليتر من بوست برايمري بلوك مع 1 ميكروليتر من البروتيين بلوك وتركناهم لمدة ساعة على 4م°، ومن ثم قمنا بغسل الفتحات 6 مرات ب300ميكروليتر من PBS مع التووين، ثم أضفنا نقطة من النوفولينك بوليمر على كل فتحة وتركناهم لمدة 30 دقيقة على درجة حرارة الغرفة لتحفيز الإستبيان، ومن ثم قمنا بغسل 6 مرات ب300ميكروليتر من PBS مع التووين، ومن بها أضفنا 3 ميكروليتر من 3,3′5, 5′-tetramethylbenzidine hydrogenperoxide في الفتحات التالية أ1، أ2، أ3، ج1،ج2، وأضفنا 3 ميكروليتر من DAB Chromogen في الفتحات أ4، أ5، ج3، ج4 و لاحظنا أن اللون في الفتحات أ1، أ2، أ3، ج1، ج2 قد تغير من اللون الشفاف ألى اللون الأزرق وبعد 30 دقيقة لاحظنا أن اللون الأزرق تحول إلى اللون الأصفر وهذا يعني أنه يوجد مركب وأننا الحمدلله نجحنا في البروبوكول وفي تجربتنا.

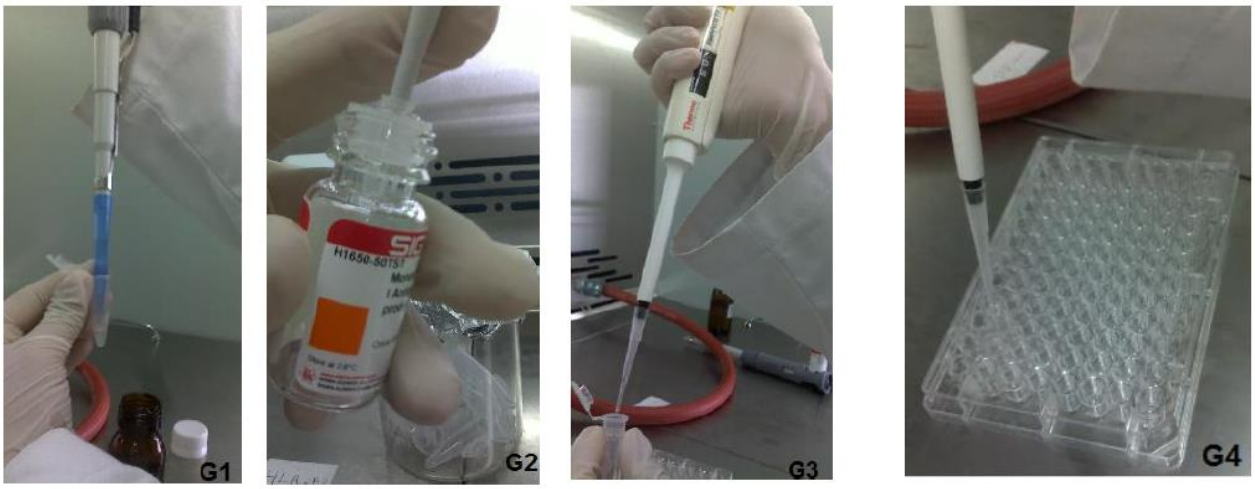


Figure 27: G3) dilute the w6/32 (G2) with the carbonate buffer (G1) and put it the wells as G4.

الصورة 27: ز3) خفف **w6/32** (ز2) مع منظم الكاربونات (ز1) ضعهم في الفتحات كما في ز4).

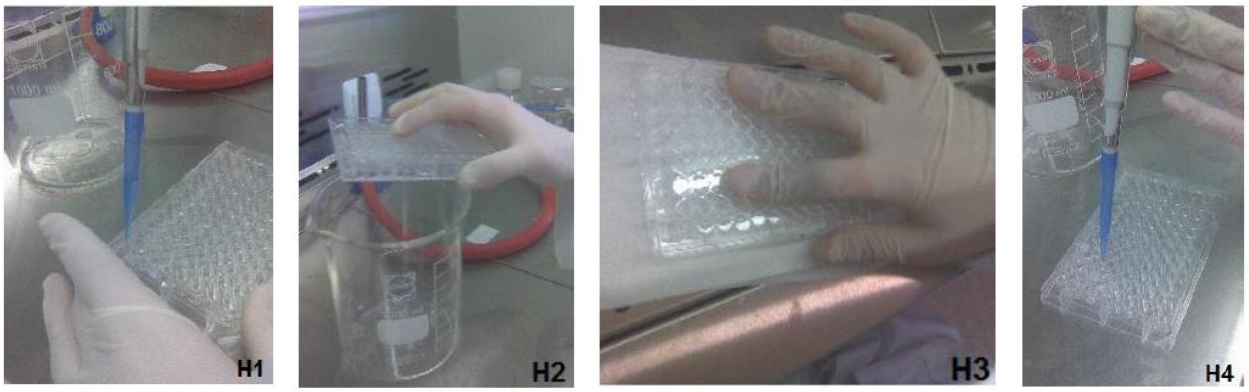


Figure 28: H1) put PBS with tween for the washing and discard them the waste as H2) then dry it by a towel as H3) then repeat the washing as the same method.

الصورة 28: ح1) ضع **PBS** مع التوين للغسل ثم إرميه في المهملات كما في ح2) ثم جففه على المنشفة كما في ح3) ثم أعد عملية الغسل على نفس الطريقة.

Figure 29: add the protein bock then the post primary block then the Novolink polymer but after adding each reagent should be wash by PBS with tween.

الصورة 29: أضف البروتين المانع ومن ثم البوست برايميري بلوك ومن ثم النوفولينك بوليمير ولكن بعد إضافة كل عامل يجب الغسل بال**PBS** مع التوين.

Figure30: add drop of the Novolink polymer in each well as J0) then add in part of wells TMB (J2) and in the second part DAB (J3), J4) observe the wells that have TMB change it color to blue after many seconds.

الصورة 30: أضف قطرة من النوفولينك بوليمير في كل فتحة كما في ي1) ثم أضف في قسم من الفتحات الTMB (ي2) وفي القسم الثاني DAB (ي3)، ي4) لاحظ أن الفتحات التي تملك TMB تغير لونها إلى الأزرق بعد ثواني

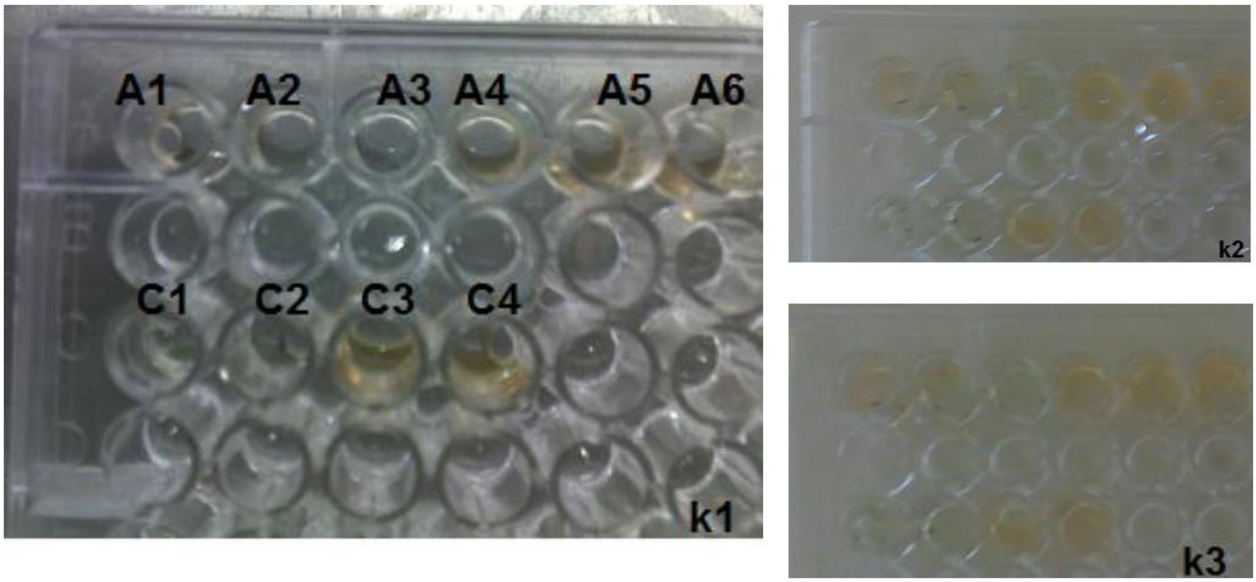


Figure 31: observe the result after 30 min the color blue change to yellow in the wells A1, A2, A3, C1, C2, that have the TMB.

الصورة 31: لاحظ النتيجة بعد 30 دقيقة اللون الأزرق تغير إلى الأصفر في الفتحات التي تملك TMB :أ1، أ2، أ3، ج1، ج2.

Appendix: *2.8*

Step 1: Amplification of HLA-A0201 and β2-m gene from PBMC

1-April-2011 Extraction of white blood cell from the whole blood	10:00 ⟶ 11:40
Purification of the white blood cells by kit And retrotranscription the RNA to cDNA	12:30 ⟶ 5:00
Result: cDNA Storage: 4°C	

2-April-2011	9:42 ⟶ 2:30

Amplification of the DNA by PCR for the HLA-A0201 and β2-m and the nested PCR for HLA-A0201	
Result: DNA amplified impure Storage: -20°C	

4-April-2011 Preparation of the solutions (TAE Buffer, Tris base)	9:00 ⟶ 12:30
5-Apirl-2011 Purification on Agarose gel	8:00 ⟶ 12:30
Gel purification by Qiaquick gel extraction kit	2:00 ⟶ 3:30
Result: DNA amplified pure Storage: -20°C.	

Step 2: Cloning of the HLA-A*0201 and β2-m gene.

7-April-2011 Preparation of vector	9:50 ⟶6:30
Result: treated vector Storage: -20°C	
8-April-2011 Ligation	**8:00 ⟶ 1:00** **With the over night**
Growing the E.coli in the LB.medium	**1:30⟶ 2:30** **With the over night**
29-April-2011 Transformation with e.coli top10 Plating on LB agar Incubation the e.coli that from hospital in lb medium	11:40⟶4:30
30-April-2011 Transformation pET into e.coli top 10 and plating onto LB plate.	10:00⟶2:00
Putting the e.coli that is form the hospital in 5ml LB medium	09:00⟶11:00

1-May-2011 Competent cell the strain that is from the hospital	10:00⟶12:00
Transformation the pET and the HLA-A*0201 into this strain	10:00⟶12:00

Plating on LB plates	11:00⟶12:00
2-May-2011 Screening the pET.	9:00 ⟶ 9:30 Incubation from 12-16 h
Preparation of 5ml of LB medium and putting the e.coli top 10 in it.	9:30 ⟶ 10:15 Incubation over night.
3-May-2011 Purification the pET by Qia prep kit.	11:30 ⟶ 12:30
Competent cells for the e.coli top 10	1:00 ⟶ 3:00
5-May-11 Competent cells for top 10 Transformation Plating on lb agar	9:00 - 2:00

6-May-11 Preparation of PMSF Screening the colonies in 2.5ml with 1.25µl	9:00 ⟶ 3:30
7-May-11 Preparation of PMSF	9:00 ⟶ 2:00
9-May-11 Screening the colonies in 2.5ml LB medium with 1.25µl ampicillin. Preparation of PMSF	9:00 ⟶ 2:00
10-May-11 Preparation of PMSF Plate the bacteria that did for it the screening	9:00 ⟶ 2:00
11-May-11 Screening the colonies	9:00⟶ 10:50
12-May-11 Lysis of the bacteria Dissolve the protein Purification with chromatography QFF	9:00 ⟶ 7:40
13-May-11 The first day in ELISA test: preparation the coated buffer with the carbonate buffer and	10:00 ⟶ 11:50

we putted it into the wells plate	
14-May-11 The second day of ELISA test: preparation of PBS, tris maleat buffer and we make the complex HLA-A0201 and the peptide	9:00 ⟶ 1:00 Overnight 48hours
15-May-11 Incubation and washing in the well plate of ELISA test until arriving to the result.	12:40 ⟶ 6:10

2.9 References

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[xiv] http://sciencetrader.com/SMF/index.php?topic=44.0

[xv] United States & Canada 800-207-0144,Germany 0800 6931 000,United Kingdom 0800 622935,Or your local sales office,www.novagen.com

Basics: Biotechnological upstream and downstream processing, DNA vaccine technology, Hepatitis B vaccine production technology[32]

[32] From [MEGBI-VPP 2012] and [MEGBI-VPP 2013]

مدخل تطبيقي الى البيوتكنولوجيا

تكثير خلايا في داخلها جينات يراد انتاج بروتينها وتنقية هذه البروتيئينات

Introduction to Biotechnological upstream and downstream processing

جميع التفاصيل باللغة العربية والمصطلحات العلمية باللغتين العربية والانجليزية

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آخر تعديل 2020/08/31

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الشركة اللبنانية الالمانية للبيوتكنولوجيا

رقم ٣٩٩ في سجل التجاري بيروت مسجل في تاريخ ٢٠٠٩/٥/٢٨

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المضمون

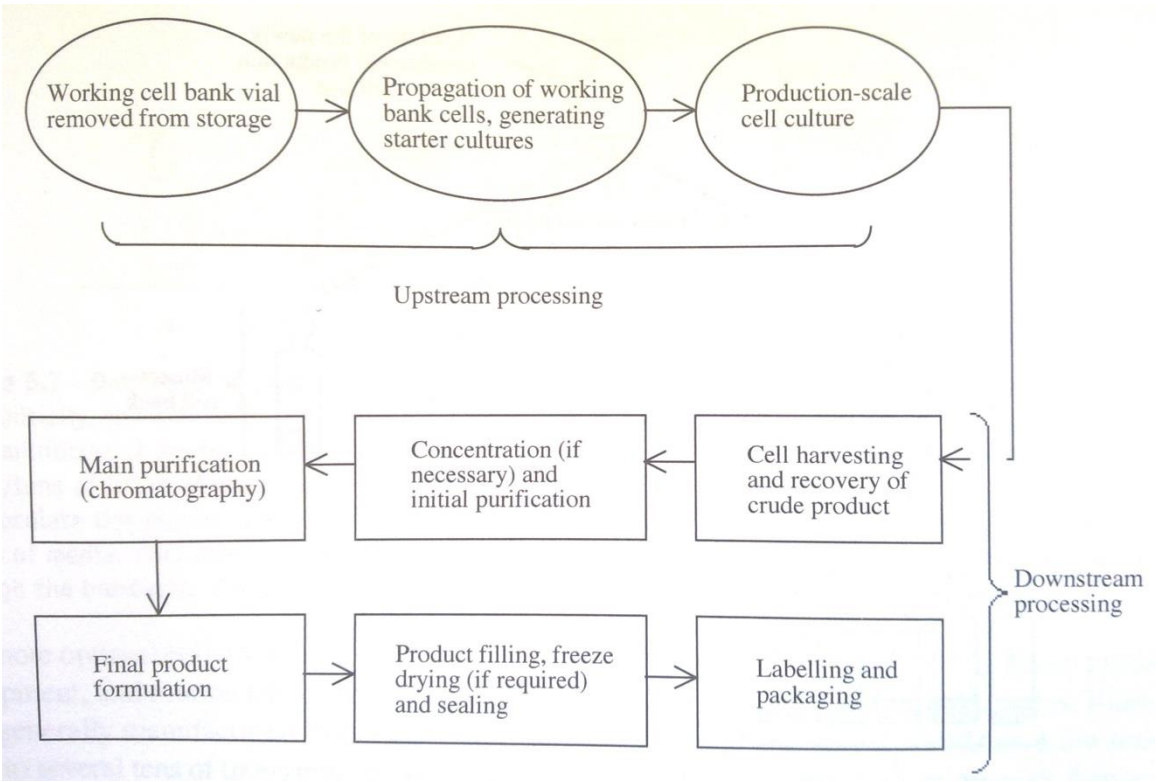
3 نظرة عامة في عملية انتاج بروتينات علاجية في البيوتكنولوجيا

ان انتاج الجزيئات الحيوية ينقسم الى معالجة المنبع (upstream) و المصب (downstream).

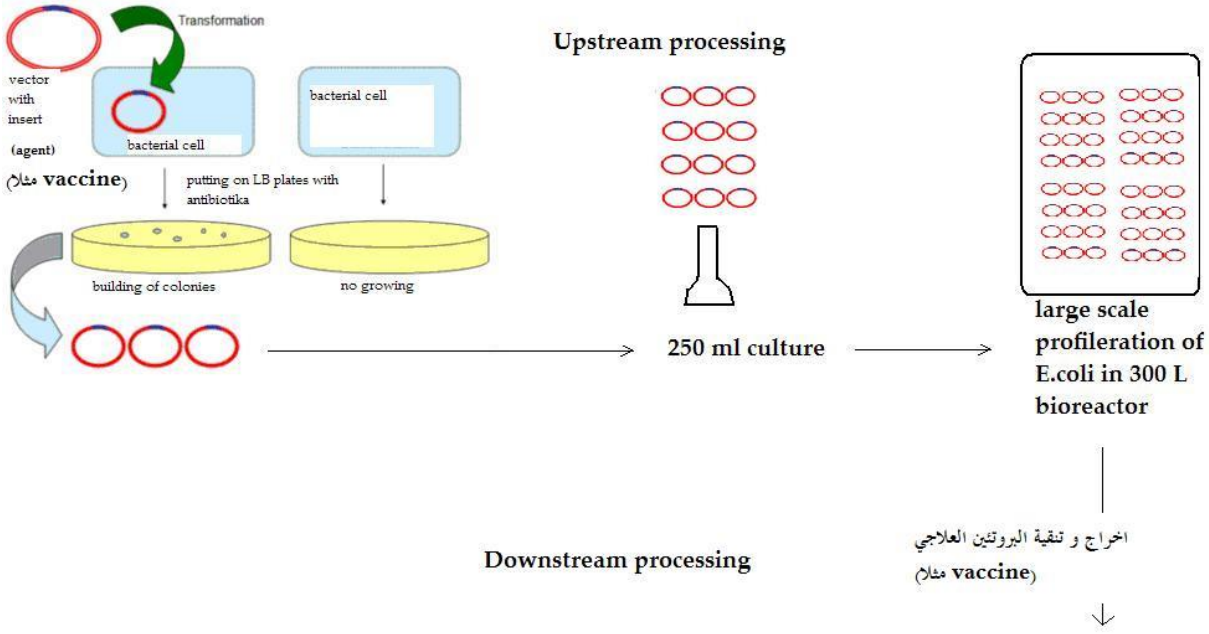
المنبع عبارة عن جزء من العملية الحيوية (upstream bioprocess) وهو أول خطوة لنمو الجزيئات الحيوية عن طريق المفاعلات الحيوية التي تحدث بواسطة خطوط الخلايا البكتيرية أو الحيوانية. وعندما تصل إلى الكثافة المطلوبة يتم حصدها ثم نقلها إلى المصب (downstream) وهي قسم آخر من العملية الحيوية.

المعالجة المتعاقبة للنواتج النهائية لعملية التخمر (downstream processing) : هي تنقية المواد الكيميائية، والمستحضرات الصيدلانية والمكونات الغذائية الناتجة عن التخميرأو الاصطناع في الأنسجة النباتية والحيوانية، مثل المضادات الحيوية، وحمض الليمون، وفيتامين(E) ، والأنسولين.[33]

الصورة مأخوذة في الاسفل مأخوذة من [Walsh 2007].



[33] من http://ar.wikipedia.org/wiki/%D9%85%D9%84%D8%AD%D9%82:%D9%82%D8%A7%D8%A6%D9%85%D8%A9_%D8%B7%D8%B1%D9%82_%D8%A7%D9%84%D8%AA%D9%86%D9%82%D9%8A%D8%A9_%D9%81%D9%8A_%D8%A7%D9%84%D9%83%D9%8A%D9%85%D9%8A%D8%A7%D8%A1



الصورة فوق: مثال

4 مدخل الى تكثير خلايا في داخلها بروتأينات يراد انتاجها (sources and upstream processing)

4.1 مصادر لانتاجات بيوصيدالية (sources of biopharmaceuticals)

4.1.1 إي كولاي (E. coli) كمصدر لبروتين (protein) علاجي مدمج (recombinant)[34]

تمثّل العديد من الكائنات الحيّة المجهرية أنظمة إنتاج جذّابة للبروتين العلاجّي. يمكن أن يكونوا مثقّفين كالعادة في كميات كبيرة، بشكل رخيص وفي مدّة قصيرة، بالطرق القياسية من الإختمار. وسائل إنتاج يمكن أن تبنى في أيّ منطقة عالمية، وسلم الإنتاج يمكن أن يغيّر كما هو مطلوب.

تعبير بروتين المدمج في الخلايا التي لا يحدثون فيها طبيعيا و إنتاج بروتين معيّنون مختلفون . الأدوية البيولوجية الأولى المنتجة بهندسة الجينات (genetic engineering) لكسب الموافقة التسويقية (في 1982) كانت الأنسيولين المدمج الإنساني (recombinant human insulin) (اسم تجاري humulin) أنتج في إي. كولاي (E. coli). مثال لأكثر دواء بيولوجي مصدّق مؤخراً الذي أنتج في إي. كولاي المدمجة هو الذي من keratinocyte kepivance , يستعمل لمعالجة معدل نمو mucositis ، و يوجد العديد من الأمثلة الإضافية.

كنظام إنتاج الدمج ، إي. كولاي يعرض عدد من الفوائد. تتضمن :

1. عمل إي . كولاي (E. coli) كنظام نموذجي لمدة طويلة في الدراسات التي تتعلّق بعلم وراثة بروكاريوتيك (prokaryotic). أنه علم الأحياء الجزيئي الذي ميز جيدا
2. مستويات عالية من تعبير (expression) بروتين (protein) مختلفة يمكن أن تنجز في إي. كولاي المدمج (recombinant E. coli). حاليا، مروّجو التعبير العاليين الحديثين يمكن أن يضمنوا بشكل دوري وصول تلك المستويات من تعبير بروتين مدمج بحدود نسبة 30 بالمائة بروتين خلوي كلي.
3. خلايا إي كولاي تنمو بسرعة على أجهزة بسيطة و رخيصة نسبيا، وتقنية الإختمار الملائمة المبنية جيدا.

[34] المضمون معظمه من [Walsh 2007], Ch5

هذه الفوائد وخصوصا سهولة التلاعب الوراثي بها، جعل من إي. كولاي (E. coli)، نظام إنتاج أساسي للادوية البيولوجية لعدّة سنوات.

على أية حال، إي. كولاي (E. coli) أيضا عرضة لعدد من العوائق كالادوية البيولوجية المنتجة. هذه تتضمن:

- تجمّع بروتين هيترولوجووس (heterologous) داخل الخلية
- عدم قابلية لتعهّد تعديلات ما بعد الترجمة (translation) (خصوصا glycosylation[35]) للبروتين
- وجود ليبوزاخاريد (lipopolysaccharide) (إل بي إس) على سطح إي. كولاي (E. coli).

معظم البروتينات التي تنتج طبيعيا هي داخل خلية إي.كولاي (E.coli) (مثلاً: البروتينات المتشابهة (homologous proteins)[36]). بعض البروتينات تفرز خارج الخلية . البروتين المركب المصنع داخل إي.كولاي يتجمع دائما في سيتوبلاسم الخلية . البروتين المصنع داخل الخلية يعقد معالجته النهائية عند تنقية البروتين (نسبة إلى الإنتاج خارج الخلية) مثال :

- يتطلب خطوات معالجة أساسية إضافية، وبمعنى آخر:تنقية الخلية بالإزالة اللاحقة من حطام الخلية بتقنية التثفيل (تنبيذ) (centrifugation) أو التنقية ؛
- تنقية أكثر شمولية بواسطة ال chromatographic لكي يفصل البروتين المهم من عدّة آلاف بروتين متشابه إضافي أنتج من قبل خلايا إي.كولاي (E.coli).

[35] **Glycosylation** is the reaction in which a carbohydrate, i.e. a glycosyl donor, is attached to a hydroxyl or other functional group of another molecule (a glycosyl acceptor). In biology glycosylation refers to the enzymatic process that attaches glycans toproteins, lipids, or other organic molecules. This enzymatic process produces one of the fundamental biopolymers found in cells (along with DNA,RNA, and proteins). Glycosylation is a form of co-translational and post-translational modification. Glycans serve a variety of structural and functional roles in membrane and secreted proteins. (from: edited by Ajit Varki ... (2009). Essentials of Glycobiology. Ajit Varki (ed.) (2nd ed.). Cold Spring Harbor Laboratories Press. ISBN 978-0-87969-770-9.)

[36] Heterologous (meaning 'derived from a different organism'), homologous: similar natural protein. Protein homology is biological homology between proteins, meaning that the proteins are derived from a common "ancestor". The proteins may be in different species, with the ancestral protein being the form of the protein that existed in the ancestral species (orthology). Or the proteins may be in the same species, but have evolved from a single protein whose gene was duplicated in the genome (paralogy).

تعقيد اضافي من التعبير (expression) العالي المستوى للبروتين المركب هو ادراج تشكيل جسم . إدراج أجسام (أجسام قابلة للكسر ثانية) وهي تجمعات عديمة الذوبان من منتج مركب مطوي جزئيا.

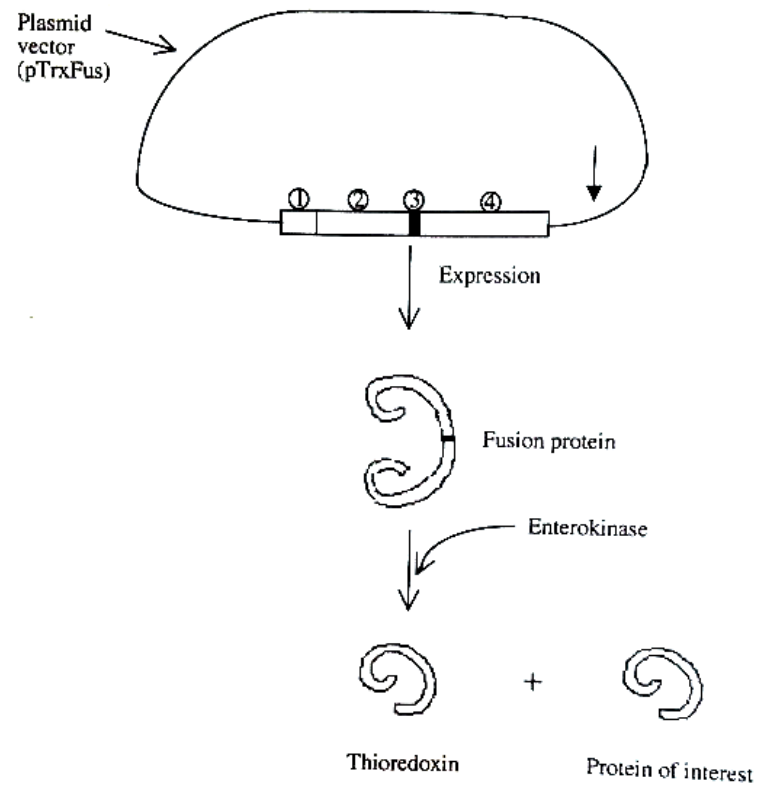
بسبب طبيعتهم الكثيفة (dense)، يلاحظون بسهولة بإستعمال مجهر الحقل المظلم (dark-field microscopy). من المفترض، عندما يعتبر (expressed) في مستوى عالي , البروتين الاجنبي (heterologous protein) يُحمل آلية طيّ البروتين الخلوية الطبيعية تحميلاً زائداً. في مثل هذه الظروف، سيكون من المحتمل اختفاء تلك الرقع الهيدروفوبية (hydrophobic) عادة من المحيط المائية المحيط بالبروتين المطوي (folded) بالكامل يبقى مكشوف في المنتج المطوي جزئيا. هذا، سيروّج التشكيل الكلي عن طريق التفاعلات (interactions) الهيدروفوبية بين الجزيئات.

على أية حال، يعرض تشكيل أجسام الإدراج (inclusion bodies) معالجة فائدة واحدة: يسهّل الإنجاز لدرجة هامّة من التنقية (purification) اللاحقة من قبل خطوة تثفيل (تنبيذ) (centrifugation) واحدة. بسبب كثافتهم العالية، تترسّب أجسام الإدراج بدرجة سرعة أكبر من حطام الخلية (cell debris). مستوى السرعة منخفض بالتثفيل (تنبيذ) (centrifugation) يسهّل للمجموعة السهلة والإنتقائية للإدراج مباشرة بعد تسوية (homogenization) خلوية (cellular). بعد التجميع ، أجسام إدراج تفقّس عموما مع denaturants قوية، مثل المنظّفات (detergents) , solvents أو urea. هذا يروّج للتذويب الكامل من جسم الإدراج (يعنى denaturation الكامل للبروتين في ذلك المكان).مادة الديناتيوريشن (denaturant) ثمّ تزال بالتقنيات مثل dialysis أو diafiltration. هذا يسهّل طوي البروتين (protein folding) ثانية، نسبة عالية منها ستطوى عموما كطيته الأساسية، ويكون نشيط حيويا، مطابق للأساسي.

محاولات المختلفة اجريت لمنع تشكيل اجسام الإدراج عندا تعبير بروتين لجين خارجي (heterologous protein) في إي. كولاي (E.coli). كشفت بعض الدراسات بأن تخفيض بسيط في درجة حرارة نمو الجرثومة (من 37 الى 30 درجة مئوية) يمكن أن ينقص حادثة تشكيل جسم الإدراج بشكل ملحوظ . كشفت دراسات أخرى تعبير البروتين الذي يهمنا كذوبان مشترك (fusion protein) مع thioredoxin سيزيل تشكيل جسم إدراج في أكثر الحالات . ذيوريدوكسين (thioredoxin) هو بروتين طبيعي لإي. كولاي (E.coli) تُعبر (expressed) فيه بمستويات عالية. هو موجود في مناطق الإلتصاق في إي. كولاي (E.coli) وهو بروتين مستقرّ (stable) على الحرارة. يوجد بلازميد (plasmid) مهندس لتسهيل تعبير البروتيين الذي يشمل (thioredoxin) المرتبط بالبروتين الذي يهمنا عن طريق سلسلة من ببتيدات (peptides) قصيرة معرفة بواسطة الإنزيم البروتياز (protease) enterokinase[37].

[37] From Wikipedia: (also called **enterokinase**) is an enzyme produced by cells of the duodenum and involved in human digestion. It is secreted from intestinal glands (the crypts of Lieberkühn) following the entry of ingested food passing from the stomach. Enteropeptidase converts trypsinogen (a zymogen) into its active form trypsin, resulting in the subsequent activation of pancreatic digestive enzymes.[1][2]

للشرح انظر الصورة في الاسفل:



Key: ① = strong promoter

② = thioredoxin gene

③ = nucleotide sequence coding for the peptide sequence which serves as cleavage site for the protease (enterokinase)

④ = gene/cDNA coding for the protein of interest

Gary Walsh, Pharmaceutical Biotechnology – Concepts and Applications, Wiley, 2007, Fig. 5.1

Enteropeptidase is a serine protease (EC 3.4.21.9) consisting of a disulfide-linked 82–140 kDa heavy chain which anchors enterokinase in the intestinal brush border membrane and a 35–62 kDa light chain which contains the catalytic subunit.[3]Enteropeptidase is a part of the chymotrypsin-clan of serine proteases, and is structurally similar to these proteins.

[1] Kunitz M (March 1939). "Formation of trypsin from crystalline trypsinogen by means of enterokinase". J. Gen. Physiol. 22(4): 429–446. doi:10.1085/jgp.22.4.429. PMC 2141988. PMID 19873112.

[2] Kiel B (1971). "Trypsin". In Boyer PS. The Enzymes, 3: Hydrolysis - Peptide Bonds. Amsterdam: Elsevier. pp. 249–275.ISBN 0-12-122703-0.

[3] Huang L, Ruan H, Gu W, Xu Z, Cen P, Fan L (2007). "Functional expression and purification of bovine enterokinase light chain in recombinant Escherichia coli". Prep. Biochem. Biotechnol. 37 (3): 205–17. doi:10.1080/10826060701386695. PMID 17516250.

إنّ البروتين الانصهاري (fusion protein) يعبر دائما في المستويات العالية، بينما يبقى في الشكل القابل للذوبان (soluble form). التجمع في مناطق الإلتصاق (adhesion zones) يسهّل إطلاقه (release) الإنتقائي (selective) إلى مستنبت (culture media) بالصدمة (shock) التنافذية (osmotic) البسيطة.

هذا ييسِّط التنقيتها (purification) اللاحقة كثيرا. بعد إطلاقه، البروتين الانصهاري يحتضن (incubated) مع enterokinase، ويُطلق البروتين الذي يهمنا (انظر الصورة فوق). وسيلة بديلة من التخفيض / ازالة تراكم جسم إدراج (inclusion body) تستلزم التعبير (expression) العالي المستوى من كابيرونس جزيئية (molecular chaperones) سويّة مع البروتين الذي يهمنا.

كابيرونس جزيئية (molecular chaperones) هم نفسهم بروتينات الذين يروّجون للطّي (folding) الصحيحة والكاملة لبروتينات و اعطائهم شكلهم الثلاثي الأبعاد (3-dimensional) النشيط حيويا (biological active). وينجزون هذا عادة بتوصيل بالبروتين الهدف أثناء المراحل المبكّرة من طيّها وتوجيه الطيّ فيما بعد بمنع / تصحيح حدوث ارتباطات هيدروفوبية (hydrophobic) غير صحيحة.

عدم قدرة البروكاريوتس prokaryotes مثل إي. كولاي لتنفيذ تعديلات ما بعد الترجمة (translation) (خصوصا glycosylation) يمكن أن يحدّد فائدتهم كأنظمة إنتاج لبعض البروتينات علاجية المفيدة. الكثير من البروتينات المشابهة، عندما تنتج طبيعيا في الجسم، تكون glycosylated (انظر القائمة في الاسفل).

البرتينات في الجدول هي glycosylated اذا انتجت طبيعياً في الجسم:[38]

اكثرية الانترلويكينس (interleukins). IL-1 مستثن من ذلك
IFN-â و IFN-ã ،(اكثر الIFN-á غير glycosylated)
CNFs
TNFs
Gonadotrophins (FSH, …)
Blood factors (e.g. Factor VII, VIII and IX)
EPO
Intact monoclonal antibodies

[38] Table 5.4 from [Walsh 2007]: Proteins of actual or potential therapeutic use that are glycosylated when produced naturally in the body (or by hydridoma technology in the case of monoclonal antibodies)

ولكن عند بعض البروتينات السكّرية (glycoproteins) عدم وجود المكون الكربوهيدرات (carbohydrate component) ،لا يؤثر سلبا على نشاطهم الحيوي. الشكل الغير glycosylated من الانترلويكين (IL-2)، على سبيل المثال، يعرض نشاط حيوي مماثل للأساسي. في مثل هذه الحالات، إي. كولاي يمكن أن تكون كافية كنظام انتاج. القلق الآخر فيما يتعلق بإستعمال إي. كولاي (E.coli) وجود جزيئيات ال بي أس (LPS) على سطحها . الطبيعة pyrogenic لإل بي إس ([Walsh 2007], Ch7) يجعل من الضروري إزالتها من المنتج. ولله الحمد تنجز إجراءات التنقية (downstream processing) المستخدمة عموما هذا الفصل بدون صعوبة كبيرة.

تعبير بروتين مؤتلف (recombinant) في أنظمة الخلايا الحيوانية (animal cell culture systems)

التقدّم التقني يسهّل التلاعب الوراثي في الخلايا الحيوانية مما يسمح إنتاج روتيني للبروتين العلاجّي في مثل هذه الأنظمة. إنّ الفائدة الرئيسية من هذه الأنظمة هي قدرتهم على تنفيذ التعديل ما بعد الترجمة (translation) للبروتين المنتج .

كنتيجة، العديد من المواد الصيدلانية الحيوية التي هي glycosylated طبيعياً تنتج الآن في الخلية الحيوانية. خلايا ال CHO و BHK أصبحا مستخدمين كثيرا بهذا الخصوص. بالرغم من أن قدرتهم لتنفيذ تعديلات بعد الترجمة (translation) تجعل إستعمالهم مرغوب / ضروري لانتاج العديد من المواد الصيدلانية، الخلية الحيوانية تعاني عدد من السلبيات.

مقارنة مع إي. كولاي، الخلايا حيوانية تتطلب غذاء معقّد جدا، وتنمو ببطئ أكثر ومعرّضة أكثر بكثير للضرر الطبيعي و أكثر عرضة للضرر المادي .في الشروط الصناعية، هذا يترجم كلفة إنتاج متزايدة. بالأضافة إلى المواد المؤتلفة (recombinant) الصيدلانية الحيوية، الخلاية حيوانية تستعمل لإنتاج المواد الصيدلانية الأخرى المختلفة المستندة حيويا. مثل اللقاحات و أجسام مضادة أحادية السلالة (monoclonal antibodies) التي تصنع بخلاية hybridoma.

سابقاً ل interferon أنتجت أيضا في خلاية lymphoblastoid معيّن (خلية Namalwa)، والتي تنتج مستويات عالية آي إف إن (IFN-α')طبيعيا.

4.1.2 خميرة (yeast)

رُكّز الإنتباه أيضا على تشكيل أنظمة إنتاج إضافية لمواد مؤتلفة (recombinant) الصيدلانية الحيوية. خلايا خميرة (خصوصا saccharomyces cerevisiae) عرض عدد من الخصائص التي جعلتهم جذّابين بهذا الخصوص. هذه الخصائص تتضمّن:

_ علم أحيائهم الجزيئي درس بالتفصيل،مما سهّل التلاعب الوراثي بهم ؛

_ معظم هذه الكائنات الحية مسجلة في الكائنات الآمنة استعمالهم بشكل عام، واستعمالهم منذ القدم في الصناعة (ومثال على ذلك: – في تخمير والخبازة)؛

_ ينمون بسرعة نسبية في مواد مغذية رخيصة نسبيا، وحائطهم الخارجي القاسي يحميهم من الضرر الطبيعي؛

_ مناسبة صناعيا – أجهزة إختمار / تقنيات متوفرة .

_ توفر تقنية تعديل البروتين بعد الترجمة (RNA translation into amino acids).

الخميرة أكّدت بالتعبير الناجح لانواع متعددة من البروتين العلاجّي.

على أية حال، هناك عدد من الأضرارعرفت عند إنتاج بروتين من جين خارجي (heterologous protein) في الخميرة. تتضمن:

1. بالرغم من أنه قادر على انتاج بروتين من جين خارجي (heterologous protein) غليكوزيلي (glycosylated) الا أن هذا الأخير عنده غليكوزيل مختلفة عن الأساسية (عندما تعزل من مصدرها الطبيعي أو عند تعبيرها في أنظمة تثقيف الخلايا الحيوانية المؤتلفة recombinant .

2. في أكثر الحالات، تبقى مستويات تعبير من جين خارجي (heterologous protein) أقل 5 بالمائة من البروتين الخلوي الكليّ. هذه النسبة أدنى جداً من مستويات التعبيرالمنجزة في إي. كولاي(E.coli) .

على الرغم من مثل هذه الأضرار المحتملة، صدّقت عدّة مواد recombinant صيدلانية حيوية للإستعمال الطبي العامّ منتجة في الخميرة (Saccharomyces cerevisiae). بشكل مثير للأنتباه، أكثر هذه المنتجات ليست glycosylated.

الجزء الاوليكوساخاريدي (oligosaccharide component) للبروتينات السكّرية (glycoproteins) التي تنتج في الخمائر تحتوي عموماً على مستويات عالية من المانُّوز (mannose). تؤدي مثل هذه الأنماط glycosylation العالية من نوع المانُّوز (mannose) عموما الى ازالتهم السريعة من مجرى الدمّ (blood stream) . مثل هذه المنتجات، يتوقع أن تكون حياتها قصيرة عندما تعطى الى البشر بشكل مباشر الى الدم عن طريق الحقن ، وبعض مكونات سكّر الخميرة يمكن أن تكون immunogenic للبشر.

4.1.3 نظام الإنتاج بالفطر (funghi)

الفطر مهم كمنتج لبروتين heterologous، فالعديد منهم يمتلك الكثير من البروتينات المهمة في الانتاج مثل α-amylase و glucoamylase. تقنية الإختمار المناسبة متواجدة . عموما، الفطر قادر على التعبير العالي المستوى لبروتينات مختلفة ،العديد منهم يبقى داخل الخلية. الإنتاج خارج الخلية للمادة الحيوية الصيدلانية ستكون مفيدة بشكل واضح من ناحية المعالجة النهائية اللاحقة. يمتلك الفطر القدرة أيضا لتنفيذ التغييرات ما بعد الترجمة (translation) للبروتين المنتج. أنماط glycosylation يمكن أن ينجز ، على أية حال، يختلف عن الأنماط المثالية التي نحصل عليها عندما يبدى البروتين السكّري في خط خليا (cell line) mammalian. و هذا يمكن أن يسبّب تخفيض مصل(serum) نصف الحياة أو تعقيدات مناعية لدى البشر.

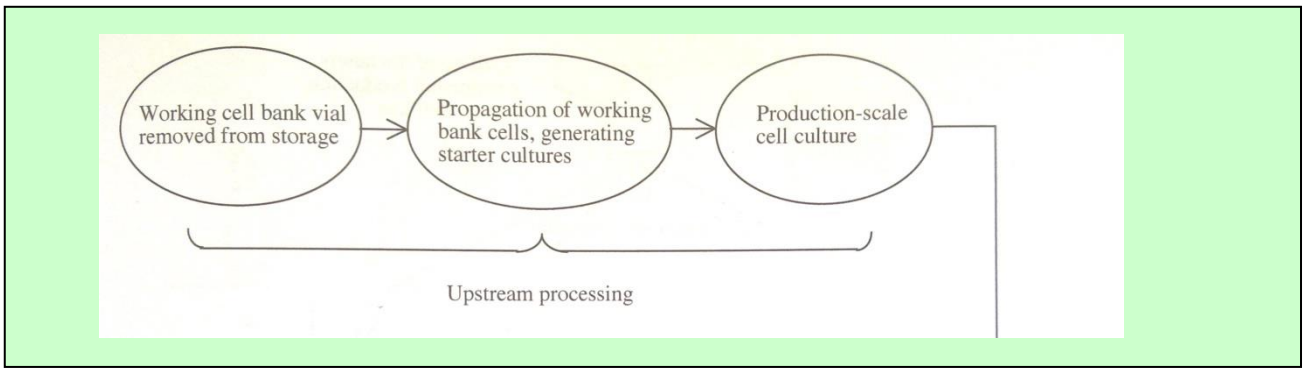
أكثر الفطريات تنتج طبيعيا كميات هامّة من الأنزيمات البروتينية الى خارج الخلية ، التي يمكن أن تخفّض (degrade) المنتج المؤتلف (recombinant). هذه الصعوبة يمكن التغلّب عليها جزئيا بإستعمال الفطري المغير جينيا (mutant) الذي يخفي المستويات المخفّضة جدا من الإنزيمات البروتينية. بالرغم من أن الباحثين أنتجوا عدد من البروتين العلاجّي المحتمل في الأنظمة الفطرية المؤتلفة (recombinant)، الا أنه لم ينتج مواد حيوية صيدلانية بمثل هذه الوسائل لهذا أرادت الحدّ و كسب موافقة تسويقية.

4.1.4 الحيوانات المعدلة وراثياً (transgenic animals)

إنتاج بروتين heterologous في حيوانات معدلة وراثياً كسب مؤخرا إنتباها كثيرا. ان انتاج جيل من الحيوانات المعدلة وراثياً (transgenic) في أغلب الأحيان نقوم به مباشرة من خلال عملية microinjecting خارجية لل دي إن أي (DNA) إلى البيض (egg).

4.2 المنبع (upstream processing)

From [Walsh 2007], Ch 5



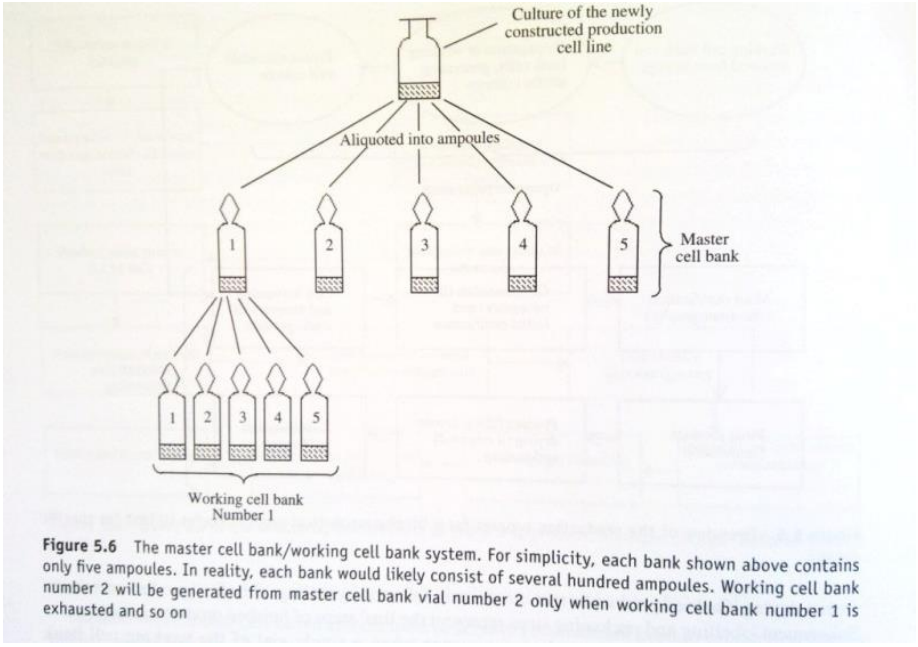


Figure 5.6 The master cell bank/working cell bank system. For simplicity, each bank shown above contains only five ampoules. In reality, each bank would likely consist of several hundred ampoules. Working cell bank number 2 will be generated from master cell bank vial number 2 only when working cell bank number 1 is exhausted and so on

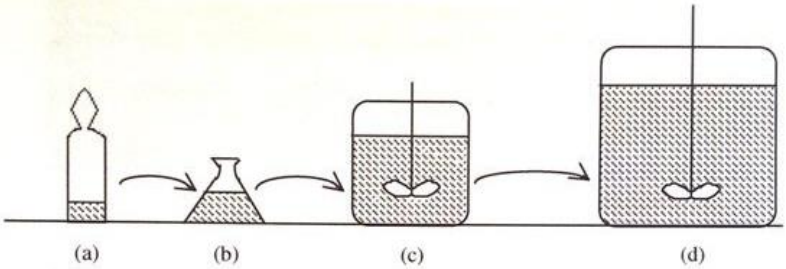


Figure 5.7 Outline of the upstream processing stages involved in the production of a single batch of product. Initially, the contents of a single ampoule of the working cell bank (a) are used to inoculate a few hundred millilitres of media (b). After growth, this laboratory-scale starter culture is used to inoculate several litres/tens of litres of media present in a small bioreactor (c). This production-scale starter culture is used to inoculate the production-scale bioreactor (d), which often contains several thousands/tens of thousands litres of media. This process is equally applicable to prokaryotic or eukaryotic-based producer cell lines, although the bioreactor design, conditions of growth, etc., will differ in these two instances

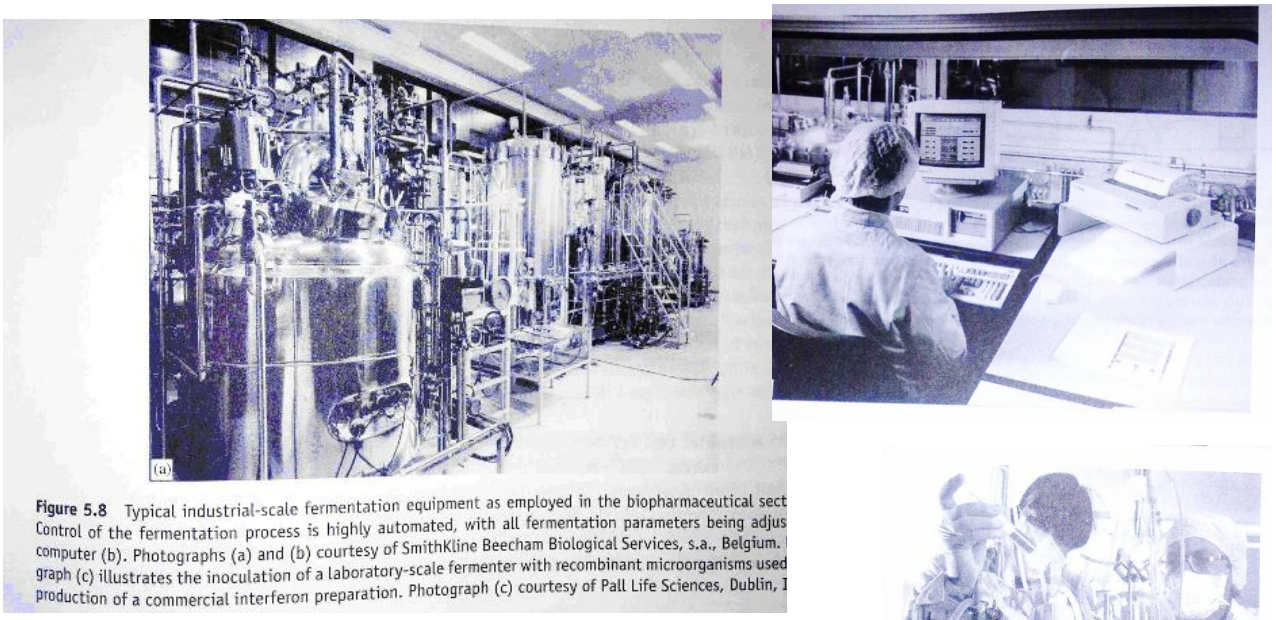


Figure 5.8 Typical industrial-scale fermentation equipment as employed in the biopharmaceutical sect Control of the fermentation process is highly automated, with all fermentation parameters being adjus computer (b). Photographs (a) and (b) courtesy of SmithKline Beecham Biological Services, s.a., Belgium. graph (c) illustrates the inoculation of a laboratory-scale fermenter with recombinant microorganisms used production of a commercial interferon preparation. Photograph (c) courtesy of Pall Life Sciences, Dublin, I

From [Walsh 2007], Ch 5

5 تحضير هلام انتقائي وتميزي (Preparation of Selective and Differential Agar)

لاعاقة (inhibit) تكاثر البكتيريا من فئة الغرام السلبي(gram negative),نحضر هلام (media) يحتوي على potassium Tellurite، Sodium azide أو Thallium acetate(0.1-0.5g/L). (tellurite agar).

لاعاقة (inhibit) تكاثر البكتيريا من فئة الغرام الاجابي(gram positive), نحضر هلام (media) يحتوي على penicillin (5-50 units/ml) أو crystal violet (2mg/L) .

5.1 أنواع الألهلام (Types of Agar)

5.1.1 هلام المانتول (Mannitol Salt Agar (MSA))

Used for the isolation of staphylococci. The pathogenic one will appear as yellow colonies with yellow zones, but the small red or pink colonies with no color changes to surrounding are the nonpathogenic.

يستعمل لعزل ال staphylococci ألخلايه المضره ستظهر كمستعمرات صفراء ومحيطها أصفر, أما الغير مضره ستكون مستعكرات صغيره باللون الأحمر أو ألوردي بدون تغير بلون المحيط.

Procedure: ألطريقه

1) 5g Enzymatic Digest of Casein
2) 1g Enzymatic Digest of Animal Tissue
3) 1g Beef extract
4) 10g D-mannitol
5) 75g Sodium Chloride
6) 0.025g Phenol red
7) 15g Agar
8) Add purified water to obtain 1L (اضافه ماء للوصول الى 1 ليتر)
9) Adjust pH to 7.4±0.2 at 25°C (ضبط ال بي أتش) .

Boil to dissolve, autoclave 15 minutes to 121°C, cool to 60°C and pouring into Petri dishes.

نغلي المزيج لتذوب المواد,نطهر لمده 15 دقيقه على حراره 121 درجه مئويه,نتركها لتبرد قليلا وتصل الى 60 درجه مئوية ومن ثم نسكبها في أوعيه خاصه.(Petri dishes)

Storage ألتخزين

Stored at 2 - 30°C.once opened and recapped, place container in a low humidity environment at the same storage temperature, protect from moisture and light by keeping container tightly closed.

ألتخزين ما بين 2 و 30 درجه مئويه. عندما تتعرض للهواء يجب وضعها بحرارة ألتخزين, ابعادها عن الرطوبه والضوء بغلق العبوه باحكام.

Packaging الطرد

Mannitol salt agar	code No	7143A	500g	
			7143B	2Kg
			7143C	10Kg

Expiration الانتهاء

Dehydrated medium should be discarded if not free following or change in color.

5.1.2 هلام الايوزن بلو (Eosin-Methylene Blue)

Used for the isolation of Gram-negative bacteria, and to differentiate between that fermenting and nonfermenting microbes of lactose and sucrose.

يستعمل لعزل الGram-negative bacteria ، و للتفرقه ما تين التي تخمر السكريات واللتي لا تستطيع.

Lactose ferment colonies were either black or dark center with colorless outer, however the non ferment are colorless.

النوع الذي يخمر يظهر على شكل مجموعات سوداء او وسطها غامق اللون, اللتي لا تخمر تكون شفافه.

Under acidic condition the dyes produce a dark purple complex associated with green metallic sheen (acidity caused by the fermentation of lactose or sucrose).

تحت تأثير الحموضه,الصباغ سيتغير للون الأخضر المعدني اللامع. و اختلاف الألوان يعود لاختلاف انواع اليكتيريا.

E.coli: dark center and a greenish metallic sheen.

Enterobacter: large, muccoid pinkish colonies.

Salmonella: non colored colonies.

Procedure: ألطريقه

1) 10g bacto peptone

2) 5g bacto lactose

3) 5g bacto sucrose

4) 2g dipotassium phosphate

5) 13.5g bacto agar

6) 0.4g bacto eosin y

7) 0.065g bacto methylene blue

8) Add purified water to obtain 1L (اضافه ماء للوصول الى 1 ليتر)

Adjust PH to 7.2, boil to dissolve, autoclave 15 minutes to 121°C, cool to 60°C and pouring into Petri dishes.

نضبط ال بي أتش، نغلي المزيج لتذوب المواد,نطهر لمده 15 دقيقه على حراره 121 درجه مئويه,نتركها لتبرد قليلا وتصل الى 60 درجه مئويه ومن ثم نسكبها في أوعيه خاصه (Petri dishes).

EMOB Agar

For testing strains of bacteria for sensitivity to phage. In this case 5g of NaCl/1L is to be added into the medium and the medium is to be made without added sugars to a final concentration of 1% as in the typical EMB.

5.1.3 هلام المكنكاي (MacConkey Agar)

Supports the growth of all *Salmonella* and *Shigella* strains and give differentiation between these enteric pathogens and the coliform group (inhibit gram positive).

Colonies of coliform bacteria are brick-red and are surrounded by a zone of precipitate bile.

Salmonella and *Shigella* do not ferment lactose but give an alkaline reaction when grow, its colonies are noncolored and transparent.

هذا الهلام يدعم تشكل مستعملرات ال *Salmonella* و*Shigella* و يميز بين ال enteric pathogens و coliform group .مجموعات ال coliform تكون أحمر قرميدي و محاطه باللون الاصفر.

مجموعات ال *Salmonella* و*Shigella* غير قادره على تخمير اللاكتوزولاكن تشكل محيط قلوي و تكون غير مرئيه.

Procedure: ألطريقه

1) 17g peptone

2) 3g proteose peptone (difco or polypeptone)

3) 10g	lactose
4) 5g	NaCl
6) 1 mg	crystal violet
7) 30 mg	neutral red
8) 1.5g	bile salts
8) 13.5g	agar

9) Add purified water to obtain 1L (اضافه ماء للوصول الى 1 ليتر)

Adjust pH to 7.1±0.2, boil to dissolve, autoclave 15 minutes to 121˚C, cool to 60°C and pouring into Petri dishes.

نضبط ال بي أتش ,نغلي المزيج لتذوب المواد,نطهر لمده 15 دقيقه على حراره 121 درجه مئويه,نتركها لتبرد قليلا وتصل الى 60 درجه مئويه ومن ثم نسكبها في أوعيه خاصه (Petri dishes)

5.2 *الفحصات البيوكميائيه* (Biochemical reactions) *اللتي تستعمل لاثبات وجود ال* gram positive cocci

5.2.1 Catalase test

A solution of dilute H_2O_2 is added to a bacterial smear on a glass slide.

The formation of bubble (O_2) is evidence of catalase activity.

$$2H_2O_2 \rightarrow 2\ H_2O$$

نضيف بضع قطرات من سائل الهيدروكسايد المخفف الى البكتيريا الموجوده على الزجاجه, اذا ظهرت فقاعات هواء فهذا دليل على قدرتها على صناعه هذا الانزيم.

آلية الفعل

السبب في تضمين الكاتالاز في خانة المواد ذات الفعل الحفزي هو قدرته على تحليل فوق أكسيد الهيدروجين إلى ماء وأكسجين جزيئي. ولكي يتم هذا التفاعل فإنه يحتاج إلى جزيئين من فوق أكسيد الهيدروجين، حيث يعمل الأول كمستقبل للإلكترونات والثاني كمعطي لها.

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

Catalase test

Catalase reaction

The catalase test is also one of the main three tests used by microbiologists to identify species of bacteria. The presence of catalase enzyme in the test isolate is detected using hydrogen peroxide. If the bacteria possess catalase (i.e., are catalase-positive), when a small amount of bacterial isolate is added to hydrogen peroxide, bubbles of oxygen are observed.

The catalase test is done by placing a drop of hydrogen peroxide on a microscope slide. Using an applicator stick, a scientist touches the colony, and then smears a sample into the hydrogen peroxide drop.

- If the mixture produces bubbles or froth, the organism is said to be 'catalase-positive'.Staphylococci[31] and Micrococci[32] are catalase-positive. Other catalase-positive organisms include Listeria, Corynebacterium diphtheriae, Burkholderia cepacia,Nocardia, the family Enterobacteriaceae (Citrobacter, E.
coli, Enterobacter, Klebsiella, Shigella, Yersinia, Proteus, Salmonella, Serratia,Pseudomonas), Mycobacterium tuberculosis, Aspergillus, and Cryptococcus.
- If not, the organism is 'catalase-negative'. Streptococcus[33] and Enterococcus spp. are catalase-negative.

While the catalase test alone cannot identify a particular organism, combined with other tests, such as antibiotic resistance, it can aid identification. The presence of catalase in bacterial cells depends on both the growth condition and the medium used to grow the cells.

Capillary tubes may also be used. A small amount of bacteria is collected on the end of the capillary tube (it is essential to ensure that the end is not blocked, otherwise it may present a false negative). The opposite end is then dipped into hydrogen peroxide which will draw up the liquid (through capillary action), and turned upside down, so the bacterial end is closest to the bench. A few taps of the arm should then move the hydrogen peroxide closer to the bacteria. When the hydrogen peroxide and bacteria are touching, bubbles may begin to rise, giving a positive catalase result.

5.2.2 اختبار الكواجيوليز (Coagulase test)

اختبار الكواجيوليز يستخدم للتفريق بين بكتيريا العنقودية الذهبية والأنواع الأخرى من بكتيريا العنقودية. الاختبار يستخدم بلازما الارنب الذي تم تطعيمة بالبكتيريا العنقودية.الانبوب يتم حضنه عند درجة حرارة 37 لمدة ساعة ونص. إذا كانت النتيجة سلبية تم اسمرار الاختبار إلى 18 ساعة..

- إذا كانت النتيجة ايجابية، البلازما سوف يتخثر ويظهر على شكل قطع متجمعه.
- إذا كانت سلبية، البلازما لايتغير.
- مجموعة من البمتيريا العنقودية موجبة الكواجليز : العنقودية الذهبية، انايروبيس ,العنقودية هايكس، العنقودية انترميدس، العنقودية لوتري..

Caogulase act by a thrombinase like action. In normal blood clotting, the following raction occur.

Prothrombin + CaCl2 → prothrombinase thrombin

Thrombin + fibrinogen → thrombinase fibrin

Coagulase acts within host tissues to convert fibrinogen to thrombin.

Add 0.5 ml of dillute rabbit plasma to a small sterile tube contain the tested bacteria and put them in a water bath to 37°C

Result: fibrin formation (clot plasma) → coagulase positive (staphylococcus aureus needs 4 hours).

العمليه تعتمد على تجمد الدم و تحويل مادة الفيبرينوجن الى ثرومبن.

نضيف 0.5 مل من بلازما أرنب مخففه الى انبوب مطّهر يحتوي على البكتيريا التي ندرسها ونضعه في حوض التسخين على 37 درجه مئويه.

اذا تجمد البلازما, فهذا دليل على قدرتها على صناعه هذا الانزيم.(تحتاج 4 ساعات S. aureus).

5.2.3 إنزيمات الحالّة الدموية (Hemolysins)

إنزيمات الحالَّة الدموية (Hemolysins) هي ذيفانات خارجية (exotoxins) تنتجها وتؤدي إلى انحلال كرات الدم الحمراء معملياً.

Test how will lyse the red blood cells.

We incubate the bacteria on a nutrient agar supplement with 5% concentration of sheep blood.

Result:

1. Alfa hemolysins: partially lyse, produce green zone.

2. Beta hemolysins: complete lysis, clearing of hemoglobin.

3. Gamma hemolysins: no lysis, no changes.

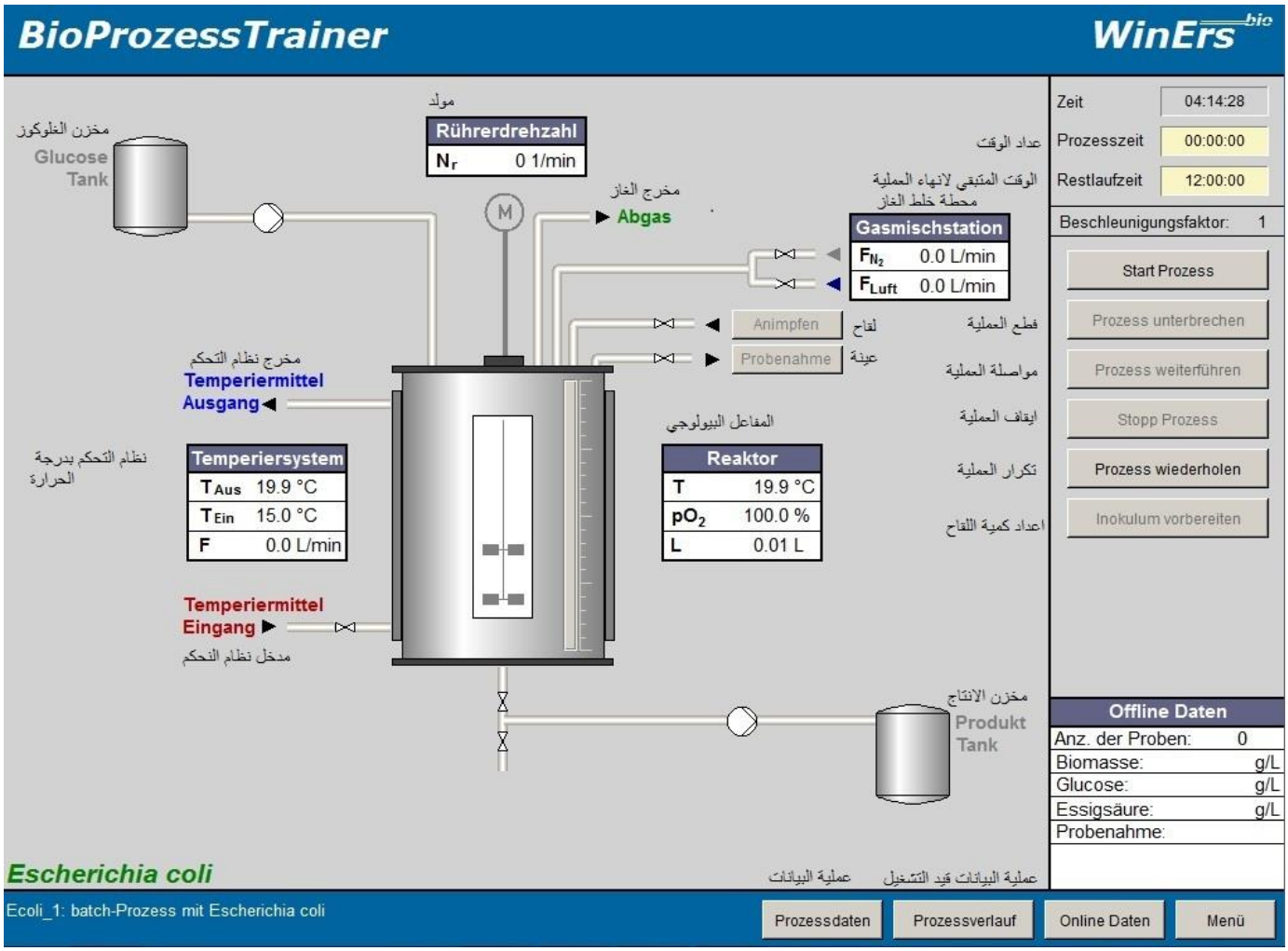
سيتم تكسير الكرويات الحمر. نزرع البكتيريا على هلام مغذ مدعم بدم خاروف بكثافه 5%.

اذا ظهر اللون الاخضر على الهلام فهذا يعني التكسير الجزئي, اذا لم يتغير اللون يعني ان الخلايه لم تتكسر, اما اذا اختفى اللون الاحمر يعني تكسير كامل.

6 تشغيل البيوریاکتور (bioreactor) - عمليات تفصيلية داخل البيوریاکتور

6.1 كيفية عمل المفاعل البيولوجي (bioreactor)[39]

الصورة في الاسفل مأخوذة من برنامج حاسوبي من [Hass, Pörtner 2011] و اضيفت اليها الترجمة العربية



للمصطلحات. بهاذا البرنامج يمكننا ان نرى ديناميك النظام على مدار الوقت اذا تم تشغيله والتدخل فيه. بالتالي سنقوم بمحاكاة (simulation) تجربة (experiment) مع إ. كولي.

بالتالي نقوم بتشغيل مثالي للبيوریاکتور

ويمكننا ان نفعل ذلك حقيقياً او بحاكات (simulation) بإستخدام برنامج BioProcessTrainer

[39] معظم مضمون هذه الفقرة من [Hass, Pörtner 2011]

أولاً : نقوم بادخال 10 ليتر من المستنبت (medium) الكلوكوز الى المفاعل .

ثانياً: نشغل المحرك الكهربائي (Ruehrer) بسرعته الأولية 50 دورة / دقيقة .

(ثالثاً : نفتح محطة دمج الغاز (Gasmischstation) ونثبت سرعة دخول الغاز الى المفاعل البيولوجي على 10 ليتر/دقيقة.)

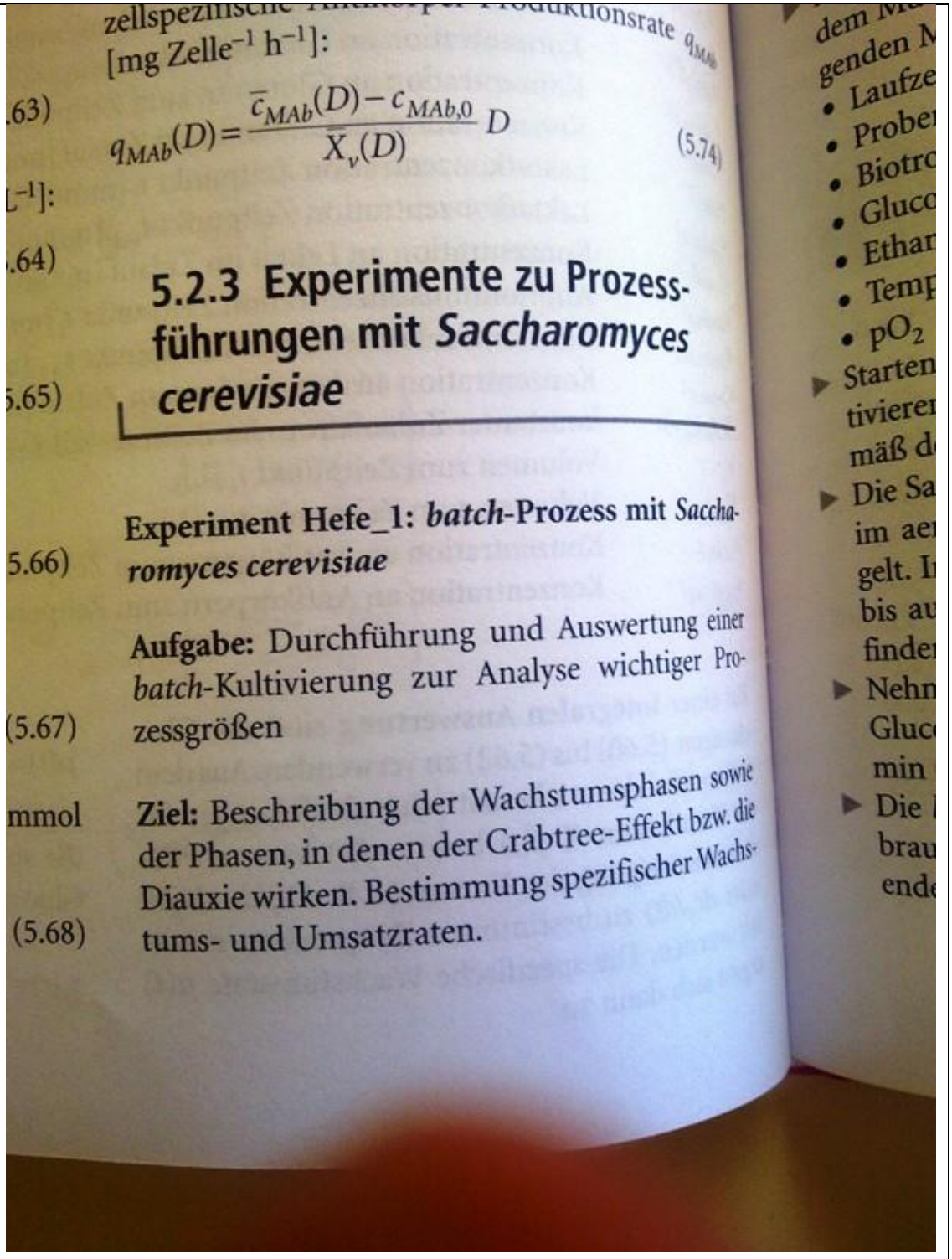
رابعاً : نشغل نظام التحكم بدرجة الحرارة (Temperiersystem) وهو عبارة عن أنبوب يلتف حول المفاعل و يدخل فيه السائل من الطرف الأسفل و يخرج من الطرف الأعلى , و يكون السائل بارداً أو ساخناً بحسب درجة حرارة المفاعل . الهدف من هذا النظام ابقاء درجة الحرارة في المفاعل ثابتة .

خامساً : نحدد كمية البكتيريا (اذا استخدمنا البنامج BioProcessTrainer: بالضغط على (Inokulum) vorbereiten)ثم ندخلها الى المفاعل البيولوجي (اذا استخدمنا البنامج BioProcessTrainer: بالضغط على (animpfen)) على أن تدخل الكمية خلال 20 ثانية .

سادساً: نأخذ عينة من المفاعل (اذا استخدمنا البنامج BioProcessTrainer: من خلال الضغط على(Probenahme). و في البرنامج على الفور تظهر كمية كل مكون من مكونات العينة في جدول المعلومات تسمى (offline daten) و هي على الشكل التالي biomasse,glucose , essigsauere)).

- الغاز الذي يدخل الى المفاعل يخرج منه عبر المخرج المسمى الAbgas.
- (اذا استخدمنا البنامج BioProcessTrainer: عندما نضغط على (online daten) خلال العملية يظهر جدول يبرز المتغيرات الحالية لكل من : معدل الحرارة المستنبت (T), معدل الحجم المستنبت (V), pH , pO2, كمية الأوكسيجين و ثاني أوكسيد الكربون (O2,CO2)).
- (اذا استخدمنا البنامج BioProcessTrainer: عندما نضغط على (Prozessverlauf) نحصل على رسم بياني لكل من : درجة الحرارة , سرعة المحرك , الحجم الاجمالي للمفاعل , ال pH و pO2, في اللحظة التي نضغط بها , هذه المعطيات نحصل عليها من خلال مجسات موصولة الى المفاعل.)
- (اذا استخدمنا البنامج BioProcessTrainer: عندما نضغط على (Prozessdaten) نحصل على رسم بياني لنفس المعطيات السابقة و لكن لفترة من الوقت و ليس للللحظة التي نضغط بها.)

6.2/اختبار مع خميرة (S.cerevisiae) مع حل ملي ببرنامج BioProcessTrainer



zellspezifische ... Produktionsrate q_{MAb} [mg Zelle^{-1} h^{-1}]:

$$q_{MAb}(D) = \frac{\bar{c}_{MAb}(D) - c_{MAb,0}}{\bar{X}_v(D)} D \quad (5.74)$$

5.2.3 Experimente zu Prozessführungen mit *Saccharomyces cerevisiae*

Experiment Hefe_1: *batch*-Prozess mit *Saccharomyces cerevisiae*

Aufgabe: Durchführung und Auswertung einer *batch*-Kultivierung zur Analyse wichtiger Prozessgrößen

Ziel: Beschreibung der Wachstumsphasen sowie der Phasen, in denen der Crabtree-Effekt bzw. die Diauxie wirken. Bestimmung spezifischer Wachstums- und Umsatzraten.

Grundlagen: Kap. 3.4 und Kap. 4.1
Auswertungsgleichungen: Kap. 5.2.2.2

Einstellungen am BioProzessTrainer:

- Wählen Sie aus dem Hauptmenü das Experiment **Hefe_1**. Hierdurch wird der **BioProzessTrainer** initialisiert. Nach der Initialisierung befinden sich $V_R = 10$ L einer auf 35 °C temperierten und mit Sauerstoff gesättigten ($pO_2 = 100\,\%$) Mediumslösung im Reaktor. Die Anfangskonzentrationen für Glucose und Ethanol betragen:
  - Glucose: 10 g L^{-1}
  - Ethanol: 0 g L^{-1}

Die Konzentration an Biomasse X_R nach dem Animpfen soll bei 4 g L^{-1} liegen.

- Berechnen Sie die erforderliche Biomassekonzentration X_I im Inokulum (Volumen Inokulum $V_I = 200$ mL)

$$X_I = X_R \frac{V_R + V_I}{V_I} \qquad (5.75)$$

Vorgehensweise:

- Bereiten Sie ein Datenblatt gemäß beiliegendem Muster (siehe Beispiellösung) für die folgenden Messgrößen vor:
  - Laufzeit t
  - Probenvolumen (hier 10 mL)
  - Biotrockenmassekonzentration
  - Glucosekonzentration
  - Ethanolkonzentration
  - Temperatur
  - pO_2
- Starten Sie das Experiment **Hefe_1** durch Aktivieren des Start-Buttons (aerob/anaerob) gemäß den Hinweisen auf der DVD.
- Die Sauerstoffkonzentration im Medium wird im aeroben Fall bei 60 % Luftsättigung geregelt. Im anaeroben Fall wird die Luftsättigung bis auf 0 % absinken. Hinweise zur Regelung finden sich in Kap. 6.
- Nehmen Sie Proben (zu Biotrockenmasse, Glucose und Ethanol) im Abstand von ca. 30 min (Prozesszeit).
- Die *batch*-Kultur ist mit dem kompletten Verbrauch an Substraten (Glucose, Ethanol) beendet.
- Tragen Sie die Daten für die Messgrößen in die vorbereitete Tabelle gemäß Musterlösung ein.
- Wiederholen Sie das Experiment unter anaeroben Bedingungen. Zur Wiederholung des Teil-Experiments drücken Sie den Wiederholungs-Button entsprechend den Hinweisen auf der DVD.
- Zum Beenden des Experiments **Hefe_1** drücken Sie den Ende-Button entsprechend den Hinweisen auf der DVD.

Auswertung:

- Stellen Sie aus den Rohdaten die Verläufe von Biotrockenmasse, Glucose- und Ethanolkonzentration als Funktion der Zeit dar.
- Unterteilen Sie den Verlauf in die exponentielle Phase (aerobes Wachstum mit Crabtree-Effekt bei Glucoseüberschuss und Ethanolbildung, Diauxie bei niedrigen Glucosekonzentrationen und Ethanolverbrauch).
- Berechnen Sie in den jeweiligen Zeitintervallen zwischen zwei Probenahmen die im Folgenden aufgeführten Größen und stellen Sie diese ebenfalls als Funktion der Zeit dar.
  - spezifische Wachstumsrate μ
  - Verdopplungszeit t_D
  - spezifische Substrataufnahmerate für Glucose q_{Glc}
  - spezifische Substrataufnahmerate für Glucose q_{Glc} und Ethanol q_{Eth} (bei Glucoselimitierung)
  - spezifische Ethanolbildungsrate $q_{p,Eth}$ bei Glucoseüberschuss
  - Ausbeutekoeffizient Biotrockenmasse/Glucose $Y_{X_{TG}/Glc}$ unter den verschiedenen Prozesszuständen (vgl. Kap. 3.4)
  - Ausbeutekoeffizient Biotrockenmasse/Ethanol $Y_{X_{TG}/Eth}$ unter den verschiedenen Prozesszuständen (vgl. Kap. 3.4)

  (Vorbereitung für Experimente in Kap. 5.3)
- Vergleichen Sie dabei die differentielle und die integrale Methode zur Bestimmung der genannten Kenngrößen.

Beispiellösung zu HEFE_1

Experiment Nr	HEFE_1	aerobe Prozessführung
Name	HEFE_1	
Organismus	*Saccharomyces cerevisiae*	

Startvolumen	10,0 L
Anfangskonzentration Glucose	10,0 g/L
Anfangskonzentration Ethanol	0,0 g/L
Animpfkonzentration	4,0 g/L
Temperatur	35,0 °C
Sauerstoffgehalt	60,0 %

Probe #	Laufzeit *t* [min]	Laufzeit *t* [h]	Biotrockenmasse *X* [g/L]	Glucose *G* [g/L]	Ethanol *E* [g/L]	Temperatur *T* [°C]	pH pH [–]	pO_2 pO_2 [%]
1	5,00	0,08	4,1	9,4	0,2	34,9	6,9	60,0
2	30,00	0,50	4,7	7,2	0,8	35,0	6,7	59,9
3	60,00	1,00	5,4	4,1	1,7	35,0	6,4	60,0
4	90,00	1,50	6,3	0,9	2,4	35,0	6,1	62,1
5	120,00	2,00	6,9	0,0	2,2	35,0	6,0	60,0
6	150,00	2,50	7,0	0,0	1,7	35,0	6,0	60,0
7	180,00	3,00	7,1	0,0	1,3	35,0	6,0	60,0
8	210,00	3,50	7,2	0,0	0,9	35,0	6,0	60,1
9	240,00	4,00	7,3	0,0	0,6	35,0	6,0	60,1
10	270,00	4,50	7,4	0,0	0,3	35,0	6,0	60,2
11	300,00	5,00	7,4	0,0	0,1	35,0	6,0	59,9
12	330,00	5,50	7,4	0,0	0,1	35,0	6,0	60,0
13	360,00	6,00	7,4	0,0	0,0	32,0	6,0	72,0

HEFE_1: Auswertung der Kenndaten der aeroben Kultivierung nach der differentiellen Methode

Laufzeit *t* [h]	Intervall-mitte *t* [h]	Vol. vor Probe V_i [L]	Vol. nach Probe V_{i+1} [L]	μ [1/h]	t_d [h]	q_{Glc} [1/h]	q_{Eth} [1/h]	$Y_{XTG/Glc}$ [–]	$Y_{XTG/Eth}$ [–]
0,08		10	9,99						
0,50	0,29	9,99	9,98	0,327	2,118	1,200	0,327	0,273	–1,000
1,00	0,75	9,98	9,97	0,277	2,500	1,228	0,356	0,226	–0,778
1,50	1,25	9,97	9,96	0,308	2,253	1,094	0,239	0,281	–1,286
2,00	1,75	9,96	9,95	0,182	3,812	0,273	–0,061	0,667	3,000
2,50	2,25	9,95	9,94	0,029	24,087	0,000	–0,144	–	0,200
3,00	2,75	9,94	9,93	0,028	24,433	0,000	–0,113	–	0,250
3,50	3,25	9,93	9,92	0,028	24,780	0,000	–0,112	–	0,250
4,00	3,75	9,92	9,91	0,028	25,127	0,000	–0,083	–	0,333
4,50	4,25	9,91	9,90	0,027	25,473	0,000	–0,082	–	0,333
5,00	4,75	9,9	9,89	0,000	–	0,000	–0,054	–	0,000
5,50	5,25	9,89	9,88	0,000	–	0,000	0,000	–	–
6,00	5,75	9,88	9,87	0,000	–	0,000	–0,027	–	0,000

HEFE_1: Auswertung der Kenndaten der aeroben Kultivierung nach der integralen Methode

Laufzeit t [h]	Intervall-mitte t [h]	Vol. vor Probe [L]	Vol. nach Probe [L]	μ [1/h]	t_d [h]	q_{Glc} [1/h]	q_{Eth} [1/h]	$Y_{XTG/Glc}$ [–]	$Y_{XTG/Eth}$ [–]
0,08	–	10	9,99	0,521	1,331	2,312	0,799	0,225	–0,799
0,50	0,29	9,99	9,98	0,367	1,889	1,459	0,426	0,251	–0,426
1,00	0,75	9,98	9,97	0,250	2,767	0,855	0,172	0,293	–0,172
1,50	1,25	9,97	9,96	0,172	4,024	0,487	0,027	0,354	–0,027
2,00	1,75	9,96	9,95	0,116	5,979	0,253	–0,056	0,459	0,056
2,50	2,25	9,95	9,94	0,074	9,384	0,103	–0,099	0,715	0,099
3,00	2,75	9,94	9,93	0,042	16,436	0,012	–0,116	3,417	0,116
3,50	3,25	9,93	9,92	0,019	36,439	–0,036	–0,113	–0,532	0,113
4,00	3,75	9,92	9,91	0,004	185,548	–0,051	–0,096	–0,074	0,096
4,50	4,25	9,91	9,9	–0,004	–191,610	–0,040	–0,070	0,091	0,070
5,00	4,75	9,9	9,89	–0,003	–274,205	–0,009	–0,038	0,296	0,038
5,50	5,25	9,89	9,88	0,008	90,707	0,036	–0,006	0,209	0,006
6,00	5,75	9,88	9,87	0,027	25,421	0,088	0,022	0,309	–0,022

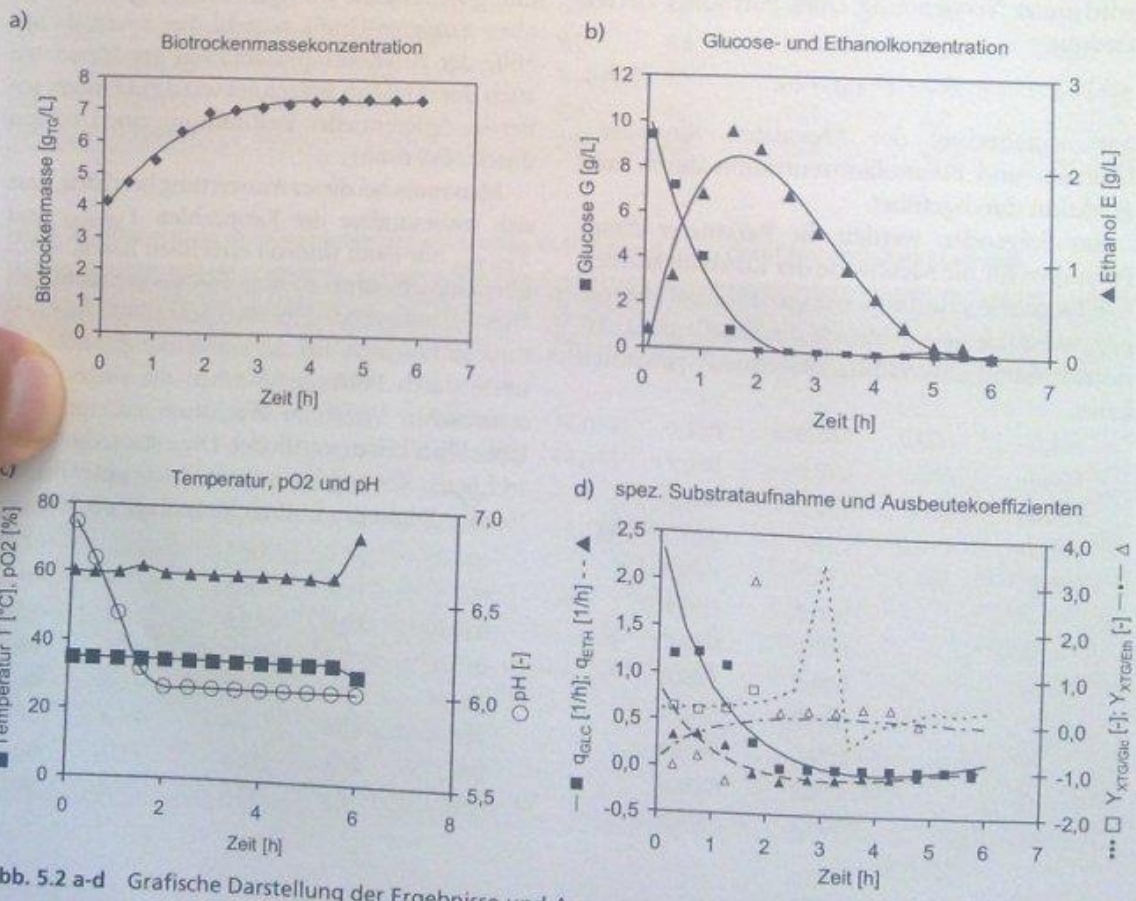


Abb. 5.2 a-d Grafische Darstellung der Ergebnisse und Auswertungen für die aerobe Kultivierung HEFE_1

In den grafischen Darstellungen der Ergebnisse und Auswertungen wurden die Kenngrößen sowohl mit der differentiellen Methode, als auch mit der integralen Methode berechnet. Differentiell erhaltene Größen sind in den Grafiken durch diskrete Symbole gekennzeichnet. Integral berechnete Größen sind in den Grafiken durch Linien verbunden (Abb. 5.2 d).

Anhand der Grafiken kann man erkennen, dass eine exponentielle Wachstumsphase für maximal 2,0 Stunden, also ungefähr bis zum Abbau der Glucose anhält. Ferner sieht man, dass der Crabtree-Effekt – also die Erzeugung von Ethanol unter aeroben Bedingungen – über den ganzen Konzentrationsbereich der Glucose zu beobachten ist. Ein Abbau von Ethanol ist erst zu beobachten, wenn die Glucose vollständig abgebaut ist.

Die integrale Berechnung von Kennzahlen wird unter Verwendung eines Polynoms vierter Ordnung

$$z(t) = a \cdot t^4 + b \cdot t^3 + c \cdot t^2 + d \cdot t + e \qquad (5.76)$$

zur Annäherung der Messdaten Biomasse-, Glucose- und Ethanolkonzentration durch eine Funktion durchgeführt.

Im Folgenden werden die Parameter dieses Polynoms für die Messwerte der Konzentrationen von Biomasse sowie Glucose und Ethanol angegeben, wie man sie z. B. durch die Tabellenkalkulationsfunktion „Trendlinie berechnen“ gewinnen kann.

	X	Glc	Eth
a	0,0015	0,0152	–0,0119
b	0,0143	–0,3716	0,2406
c	–0,3990	3,0074	–1,5830
d	2,1490	–9,7415	3,4557
e	3,8253	10,6680	–0,2621
R^2	0,9902	0,9836	0,9668

Die entsprechenden Ableitungen ergeben sich aus einem Polynom dritter Ordnung

$$\frac{dz(t)}{dt} = 4a \cdot t^3 + 3b \cdot t^2 + 2c \cdot t + d \qquad (5.77)$$

In der Abb. 5.2 d erkennt man deutlich den ausgleichenden Charakter der unterlegten Polynome. Die Kenngrößen aus der integralen Bestimmung weisen eine geringere Streuung auf (in der oben gezeigten Grafik täuscht dies etwas, da mit Hilfe der Polynomrepräsentation der Messdaten auch dort Größen berechnet werden können, wo bei der differentiellen Bestimmung eine Division durch Null droht).

Man muss bei dieser Auswertung beachten, dass sich insbesondere die Kennzahlen $Y_{XTG/Glc}$ und $Y_{XTG/Eth}$ nur dann sinnvoll errechnen lassen, wenn überhaupt Wachstum von Biomasse stattfindet. Dies ist insbesondere bei der integralen Auswertung zu beachten. Bei der Annäherung der Messwerte durch Polynome können die eingesetzten statistischen Verfahren Wachstum anzeigen, wo tatsächlich keines stattfindet. Die entsprechend errechneten Kennzahlen müssen daher unter diesen Randbedingungen kritisch hinterfragt werden.

Experiment Nr.	HEFE_1	anaerobe Prozessführung
Name	HEFE_1	
Organismus	*Saccharomyces cerevisiae*	

Startvolumen	10,0	L
Anfangskonzentration Glucose	10,0	g/L
Anfangskonzentration Ethanol	0,0	g/L
Animpfkonzentration	4,0	g/L
Temperatur	35,0	°C
Sauerstoffgehalt	0,0	%

Probe #	Laufzeit t [min]	Laufzeit t [h]	Biotrockenmasse X [g/L]	Glucose G [g/L]	Ethanol E [g/L]	Temperatur T [°C]	pH pH [–]	pO_2 pO_2 [%]
1	0,00	0,00	4,0	9,7	0,1	35,0	7,0	–0,4
2	30,00	0,50	4,1	7,8	1,0	35,0	6,8	–0,4
3	60,00	1,00	4,2	5,6	2,1	35,0	6,6	–0,4
4	90,00	1,50	4,2	3,2	3,3	35,0	6,3	–0,4
5	120,00	2,00	4,3	1,0	4,3	35,0	6,1	–0,5
6	150,00	2,50	4,3	0,0	4,8	35,0	6,0	–0,2
7	180,00	3,00	4,3	0,0	4,8	35,0	6,0	–0,2
8	210,00	3,50	4,3	0,0	4,8	35,0	6,0	–0,2
9	240,00	4,00	4,3	0,0	4,8	35,0	6,0	–0,2
10	270,00	4,50	4,3	0,0	4,8	35,0	6,0	–0,2
11	300,00	5,00	4,3	0,0	4,8	35,0	6,0	–0,2
12	330,00	5,50	4,3	0,0	4,8	35,0	6,0	–0,2
13	360,00	6,00	4,3	0,0	4,8	35,0	6,0	–0,2

HEFE_1: Auswertung der Kenndaten der anaeroben Kultivierung nach der differentiellen Methode

Laufzeit t [h]	Intervall-mitte t [h]	Vol. vor Probe V_i [L]	Vol. nach Probe V_{i+1} [L]	μ [1/h]	t_d [h]	q_{Glc} [1/h]	q_{Eth} [1/h]	$Y_{XTG/Glc}$ [–]	$Y_{XTG/Eth}$ [–]
0,00		10,00	9,99						
0,50	0,25	9,99	9,98	0,049	14,036	0,938	0,444	0,053	–0,111
1,00	0,75	9,98	9,97	0,048	14,383	1,060	0,530	0,045	–0,091
1,50	1,25	9,97	9,96	0,000	–	1,143	0,571	0,000	0,000
2,00	1,75	9,96	9,95	0,047	14,729	1,035	0,471	0,045	–0,100
2,50	2,25	9,95	9,94	0,000	–	0,465	0,233	0,000	0,000
3,00	2,75	9,94	9,93	0,000	–	0,000	0,000	–	–
3,50	3,25	9,93	9,92	0,000	–	0,000	0,000	–	–
4,00	3,75	9,92	9,91	0,000	–	0,000	0,000	–	–
4,50	4,25	9,91	9,9	0,000	–	0,000	0,000	–	–
5,00	4,75	9,9	9,89	0,000	–	0,000	0,000	–	–
5,50	5,25	9,89	9,88	0,000	–	0,000	0,000	–	–
6,00	5,75	9,88	9,87	0,000	–	0,000	0,000	–	–

HEFE_1: Auswertung der Kenndaten der anaeroben Kultivierung nach der integralen Methode

Laufzeit t [h]	Intervall-mitte t [h]	Vol. vor Probe [L]	Vol. nach Probe [L]	μ [1/h]	t_d [h]	q_{Glc} [1/h]	q_{Eth} [1/h]	$Y_{XTG/Glc}$ [–]	$Y_{XTG/Eth}$ [–]
0,00		10	9,99	0,061	11,378	1,224	0,603	0,050	–0,603
0,50	0,25	9,99	9,98	0,044	15,584	1,175	0,574	0,038	–0,574
1,00	0,75	9,98	9,97	0,031	22,081	1,045	0,508	0,030	–0,508
1,50	1,25	9,97	9,96	0,021	32,938	0,860	0,416	0,024	–0,416
2,00	1,75	9,96	9,95	0,013	53,275	0,642	0,310	0,020	–0,310
2,50	2,25	9,95	9,94	0,007	99,306	0,414	0,199	0,017	–0,199
3,00	2,75	9,94	9,93	0,003	255,019	0,197	0,095	0,014	–0,095
3,50	3,25	9,93	9,92	0,000	17060,531	0,014	0,007	0,003	–0,007
4,00	3,75	9,92	9,91	–0,001	–573,953	–0,112	–0,053	0,011	0,053
4,50	4,25	9,91	9,9	–0,001	–593,558	–0,157	–0,073	0,007	0,073
5,00	4,75	9,9	9,89	0,000	29816,419	–0,096	–0,043	0,000	0,043
5,50	5,25	9,89	9,88	0,002	311,562	0,095	0,049	0,023	–0,049
6,00	5,75	9,88	9,87	0,005	131,084	0,439	0,215	0,012	–0,215

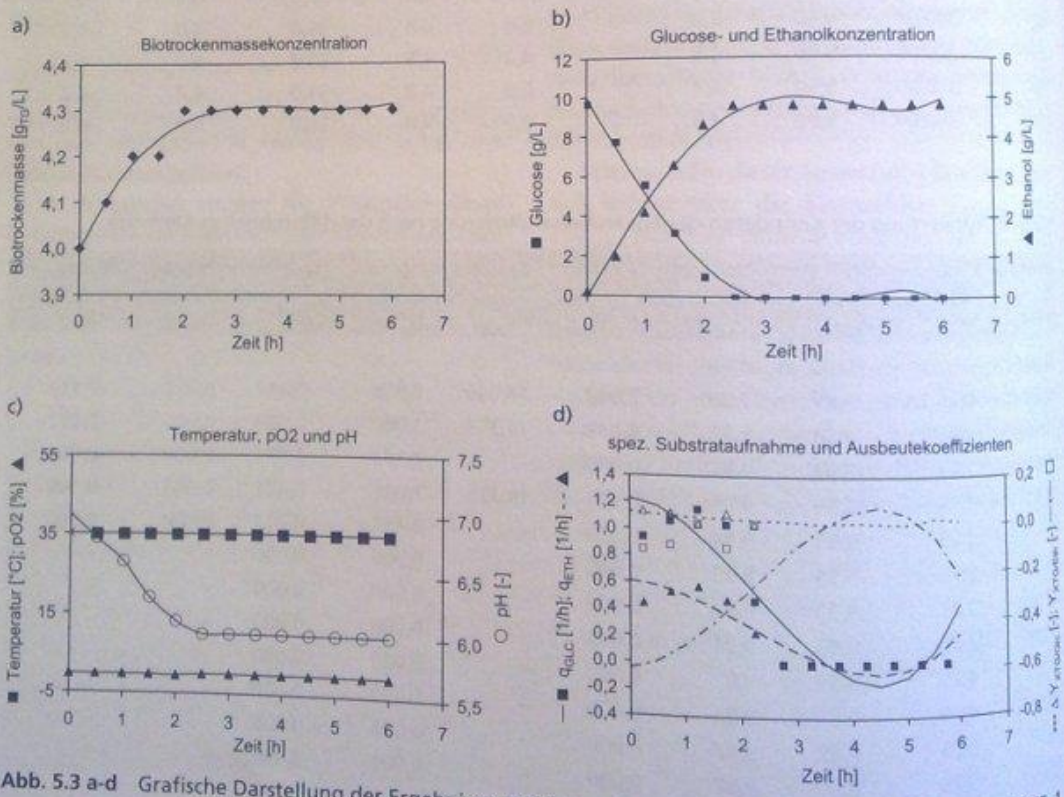
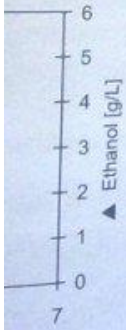
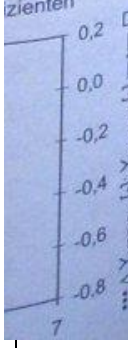


Abb. 5.3 a-d Grafische Darstellung der Ergebnisse und Auswertungen für die anaerobe Kultivierung HEFE_1

Modellbildung

$Y_{XTG/Eth}$ [–]
–0,603
–0,574
–0,508
–0,416
–0,310
–0,199
–0,095
–0,007
0,053
0,073
0,043
–0,049
–0,215





5.2 Experimente zum Wachstumsverhalten der Beispielorganismen

Die Berechnung der Kenngrößen der anaeroben Hefekultivierung erfolgt mit den gleichen Methoden wie die Berechnung der Kenngrößen der aeroben Kultivierung. Damit die Auswertungen der aeroben (Abb. 5.2) und der anaeroben Kultivierung (Abb. 5.3) einfacher zu vergleichen sind, ist die Zeitachse beibehalten worden. Es ist aber zu beachten, dass die Auswertung der Kenngrößen nur bis zum vollständigen Verbrauch der Glucose sinnvoll ist. Weil im anaeroben Betrieb dann kein Wachstum mehr erfolgt, ergeben Berechnungen von Kennzahlen, die die Wachstumsrate beinhalten keinen Sinn.

Im Vergleich der beiden Kultivierungen fällt auf, dass die Wachstumsphase bei der anaeroben Kultivierung kürzer ist als bei der aeroben Kultivierung. Die Hefezellen haben keine Möglichkeit, Ethanol zur Energiegewinnung umzusetzen. Dies zeigt sich deutlich im Ausbeutekoeffizienten Biotrockenmasse/Glucose. Dieser ist bei der anaeroben Prozessführung deutlich geringer.

Die Erwartung für den Ausbeutekoeffizienten Biotrockenmasse/Ethanol ist für die anaerobe Prozessführung Null, da kein Abbau von Ethanol zur Energiegewinnung stattfinden kann.

Experiment Hefe_2: *batch*-Prozess mit *Saccharomyces cerevisiae* bei verschiedenen anfänglichen Biomas… …bstratkonzentrationen sowie bei ver… …mperaturen

Aufgabe: Du… …Ausw… …ng von *batch*-Kultivieru… …gs-konzentrationen … verschiedenen Tem…

Ziel: Ermittlung opti… zur Erzielung einer h… tion

Grundl… …4 und Auswer… …gen:

Führen … …rim… …riment … …wobe…

Überlegen Sie sich hierzu eine … Sie mit möglichst wenigen Experim… wertbaren Aussagen kommen kön… torieller Versuchsplan [Soravia and…

Auswertung:
Bereiten Sie die Daten wie in Exper… auf und werten Sie diese entsprech…

- Welche Startzellkonzentration w… optimal ansehen?
- Welche optimalen Anfangsko… für Glucose würden Sie nach Au… Ergebnisse vorschlagen (Begrün…
- Vergleichen Sie diese Werte mit … ten.

Führen Sie ein Experiment mit den … wählten Bedingungen durch und üb… ob Ihre Prognosen zutreffen.

Experiment Hefe_3: *batch*-Prozess… *romyces cerevisiae*; Bestimmung d… und des respiratorischen Quotient… rend einer Kultivierung.

Aufgabe: Durchführung einer einf… Kultivierung mit geregeltem pO_2-… mung des k_La-Wertes in Abhängig… Rührerdrehzahl mit den *onlin*… Sauerstoffkonzentration im Abgas … löstsauerstoffkonzentration c_{O_2}.

Bestimmung des Verlaufs des res… Quotienten (*RQ*) während einer *b*… rung mit Hilfe der Abgasanalytik.

Ziel: Verfolgen des k_La-Wertes aus… ößen während einer Kultivierur… Zusammenhangs zwischen k_La-… ße Rührerdrehzahl.

…en des *RQ* während einer… *Saccharomyces cerevisiae*…

…p. 2.3

…hungen: Kap. 2.3

6.3 /اختبار دفع العملية مع ال E.coli

المهمة : اجراء و تقييم تكبير البكتيريا اذا لقح المستنبت مرة واحدة في البداية بE.coli (batch cultivation).

الهدف : وصف لمرحلة النمو، وتحديد سرعة النمو.

الاعدادات على برنامج التدريب BioProcessTrainer:

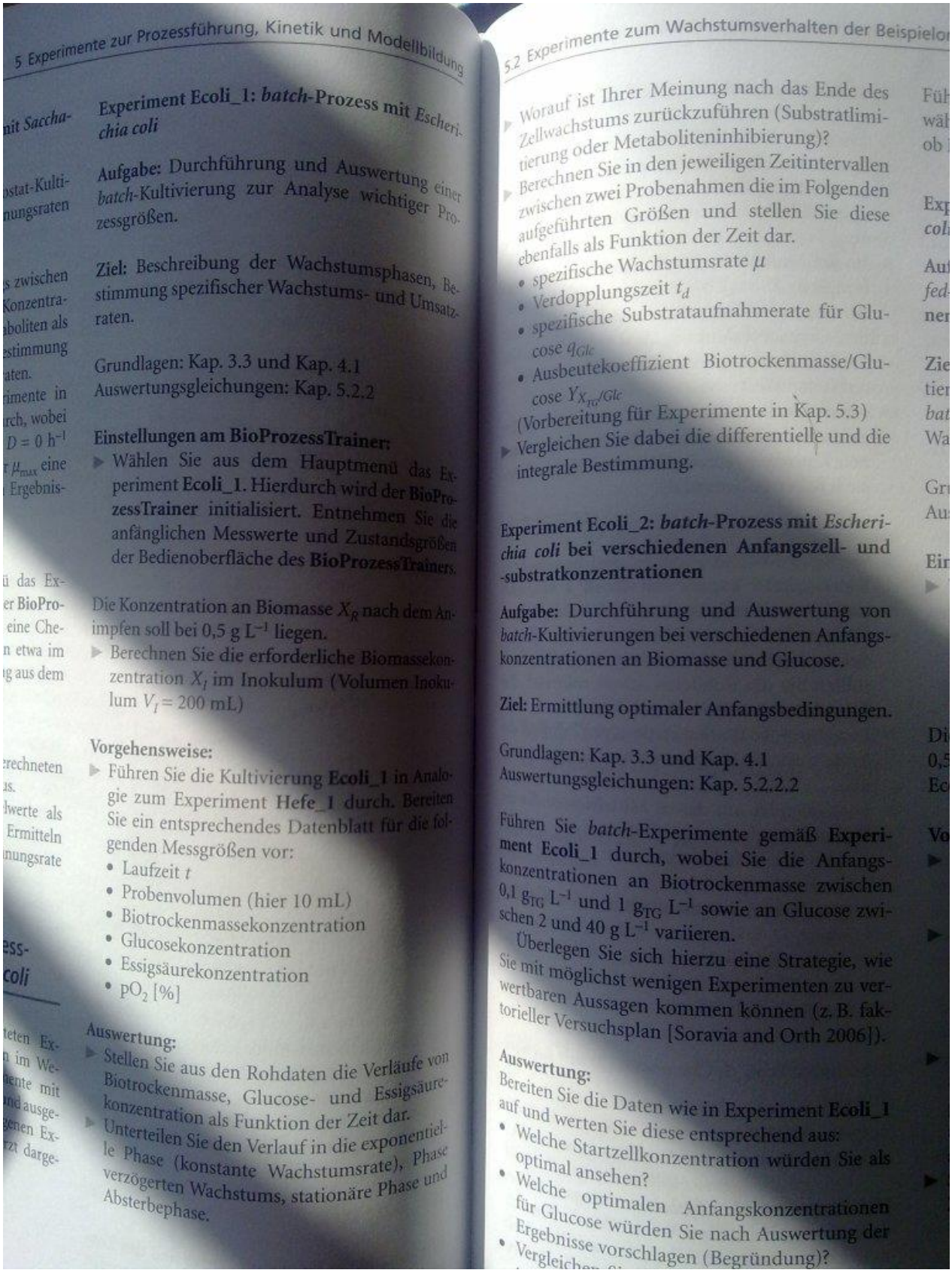
–> نختار من القائمة الرئيسية الاختبار (Ecoli_1) . و هكذا يتم استهلال (initialization) الBioProzessTrainer. خذ القيم (values) الابتدائية من شاشة التحكم لBioProzessTrainer

كثافة البيوماس (biomass) بعد التلقيح يجب ان تكون تقريباً 0.5g في الليتر.

–> احسب كثافة البيوماس (biomass) اللازمة في حجم اللقاح اذا هو يساوي 200 ml .

طريقة العمل:

عُد ورقة معطيات (data sheet) بعد قياس المتغيرات ...(انظر في الاسفل باللغة الالمانية)



5 Experimente zur Prozessführung, Kinetik und Modellbildung

Experiment Ecoli_1: *batch*-Prozess mit *Escherichia coli*

Aufgabe: Durchführung und Auswertung einer *batch*-Kultivierung zur Analyse wichtiger Prozessgrößen.

Ziel: Beschreibung der Wachstumsphasen, Bestimmung spezifischer Wachstums- und Umsatzraten.

Grundlagen: Kap. 3.3 und Kap. 4.1
Auswertungsgleichungen: Kap. 5.2.2

Einstellungen am BioProzessTrainer:

- Wählen Sie aus dem Hauptmenü das Experiment **Ecoli_1**. Hierdurch wird der **BioProzessTrainer** initialisiert. Entnehmen Sie die anfänglichen Messwerte und Zustandsgrößen der Bedienoberfläche des **BioProzessTrainers**.

Die Konzentration an Biomasse X_0 nach dem Animpfen soll bei 0,5 g L^{-1} liegen.

- Berechnen Sie die erforderliche Biomassekonzentration X_1 im Inokulum (Volumen Inokulum $V_1 = 200$ mL)

Vorgehensweise:

- Führen Sie die Kultivierung **Ecoli_1** in Analogie zum Experiment **Hefe_1** durch. Bereiten Sie ein entsprechendes Datenblatt für die folgenden Messgrößen vor:
  - Laufzeit t
  - Probenvolumen (hier 10 mL)
  - Biotrockenmassekonzentration
  - Glucosekonzentration
  - Essigsäurekonzentration
  - pO_2 [%]

Auswertung:

- Stellen Sie aus den Rohdaten die Verläufe von Biotrockenmasse, Glucose- und Essigsäurekonzentration als Funktion der Zeit dar.
- Unterteilen Sie den Verlauf in die exponentielle Phase (konstante Wachstumsrate), Phase verzögerten Wachstums, stationäre Phase und Absterbephase.

5.2 Experimente zum Wachstumsverhalten der Beispielo

- Worauf ist Ihrer Meinung nach das Ende des Zellwachstums zurückzuführen (Substratlimitierung oder Metaboliteninhibierung)?
- Berechnen Sie in den jeweiligen Zeitintervallen zwischen zwei Probenahmen die im Folgenden aufgeführten Größen und stellen Sie diese ebenfalls als Funktion der Zeit dar.
  - spezifische Wachstumsrate μ
  - Verdopplungszeit t_d
  - spezifische Substrataufnahmerate für Glucose q_{Glc}
  - Ausbeutekoeffizient Biotrockenmasse/Glucose $Y_{X_{TG}/Glc}$

(Vorbereitung für Experimente in Kap. 5.3)

- Vergleichen Sie dabei die differentielle und die integrale Bestimmung.

Experiment Ecoli_2: *batch*-Prozess mit *Escherichia coli* bei verschiedenen Anfangszell- und -substratkonzentrationen

Aufgabe: Durchführung und Auswertung von *batch*-Kultivierungen bei verschiedenen Anfangskonzentrationen an Biomasse und Glucose.

Ziel: Ermittlung optimaler Anfangsbedingungen.

Grundlagen: Kap. 3.3 und Kap. 4.1
Auswertungsgleichungen: Kap. 5.2.2.2

Führen Sie *batch*-Experimente gemäß **Experiment Ecoli_1** durch, wobei Sie die Anfangskonzentrationen an Biotrockenmasse zwischen 0,1 g_{TG} L^{-1} und 1 g_{TG} L^{-1} sowie an Glucose zwischen 2 und 40 g L^{-1} variieren.

Überlegen Sie sich hierzu eine Strategie, wie Sie mit möglichst wenigen Experimenten zu verwertbaren Aussagen kommen können (z. B. faktorieller Versuchsplan [Soravia and Orth 2006]).

Auswertung:

Bereiten Sie die Daten wie in Experiment **Ecoli_1** auf und werten Sie diese entsprechend aus:

- Welche Startzellkonzentration würden Sie als optimal ansehen?
- Welche optimalen Anfangskonzentrationen für Glucose würden Sie nach Auswertung der Ergebnisse vorschlagen (Begründung)?
- Vergleichen

7 مدخل الى تنقية البروتيئينات (protein purification) المنتوجة داخل خلايا (downstream processing)

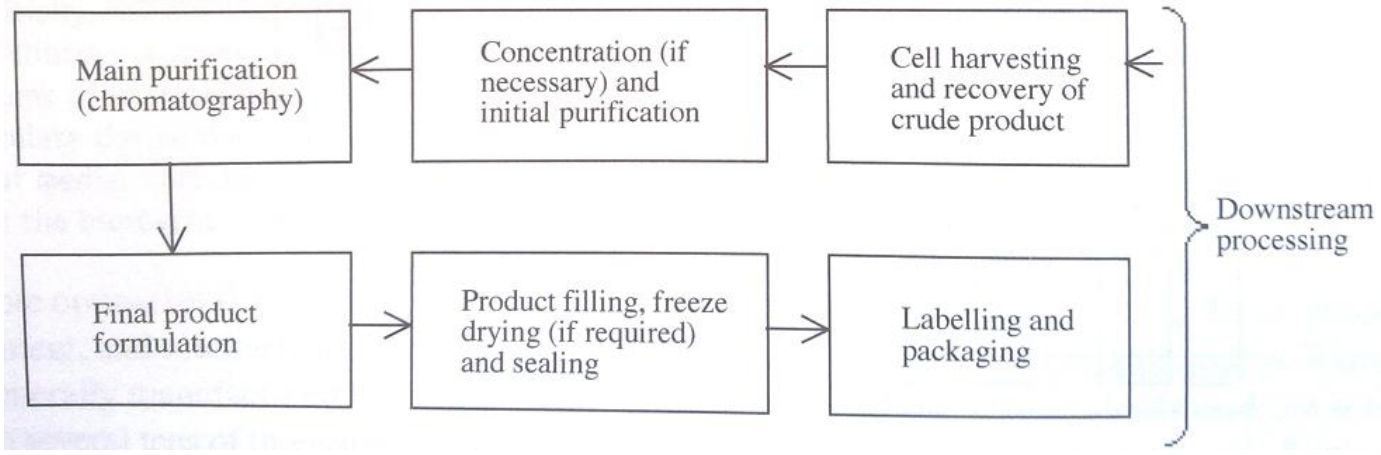


Fig.: From [Walsh 2007]

تنقية البروتين هو عبارة عن سلسلة من العمليات تهدف إلى عزل نوع واحد من البروتين من خليط معقد.

تنقية البروتين هي عملية مؤلفة من عدة مراحل : اخراج أولي , ترقيد بالاختلاف , غسيل ,كروماتوغرافي (chromatography) للأنواع المختلفة ,وغيرها

7.1 المبادئ الأساسية للتنقية

احد الطرق لتدمير الغلاف الخارجي للباكتيريا هو من خلال الصدمة الحرارية وذلك بوضعه في الثلاجة (-20°C) ومن ثم في درجة حرارة الغرفة (25°C) حتى يذوب السائل وبعدها مباشرة في الثلج مرة اخرى و تكرر الخطوات ثلاث مرات.

ومن بعدها نقوم بالتنبيذ (centrifugation) على درجة 3000 دورة في الدقيقة (rpm) فنحصل على البروتين المطلوب ولكنه موجود مختلط مع بقايا الخلية المكسورة وغيرها من مكونات البكتيريا

وهنا تبدأ التنقية الفعلية للبروتين من خلال استعمال الكبريتات الأمونيوم (ammonium sulfate) $(NH_4)_2SO_4$ كمحفز لترقيد (precipitation) البروتين وذلك من خلال جذبه للبروتين أكثر من المياه وباختلاف تركيز (concentration) الكبريتات الأمونيوم (ammonium sulfate) سيختلف البروتين المرقد وذلك بسبب جذبه المختلف وبذلك نفصل البروتين عن الأشياء الكبيرة الموجودة

ومن المفضل استعمال مرحلة تليها وهي الاستشراب (chromatography).

أحيانا بين هذه المراحل نستعمل الdialysis لنتخلص من الكبريتات الأمونيوم (ammonium sulfate) $(NH_4)_2SO_4$

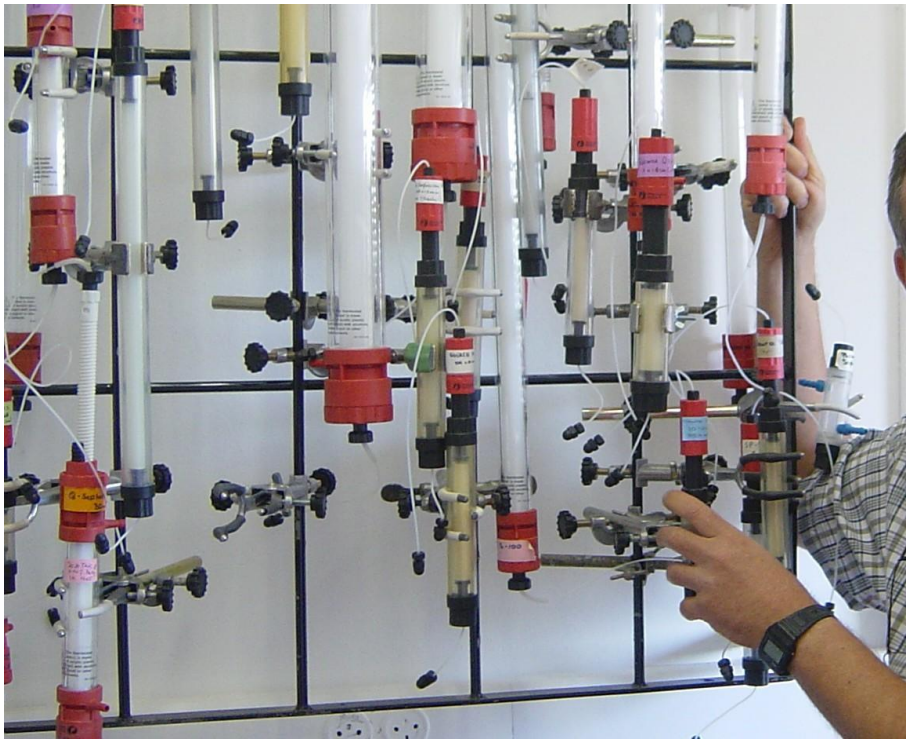
7.2 بعض الاشياء الذي يجب الانتباه اليها

الأهم في كل هذه المراحل هو الحفاظ على بنية البروتين وذلك لا يتحقق الا من خلال أن نخلق له محيط مشابه للمحيط البيولوجي الذي كان يتواجد فيه كالرقم الهيدروجيني (pH value).

ولهذا السبب سنضيف اليه NaCl 0.85 %. يعطي جو مائي قريب لذلك الذي كان متواجدا داخل الخلية. كما يجب المحافظة على الرقم الهيدروجيني pH 7.4 تقريبا وذلك من خلال اضافة PBS ("phosphate buffered saline"). ومن المهم جدا أن لا نعرض البروتين للهواء لأنه يتسبب بالأكسدة للبروتين .

ال sepharose هو مادة جاذبة توضع على chromatography column.

مثال لِ chromatography columns في نطاق مختبري (laboratory scale).



من: http://wolfson.huji.ac.il/purification/index.html

مثال لِ chromatography columns في نطاق انتاجي (production scale).

من:

GE Healthcare

Bedienungsanleitung BioProcess LPLC- und MPLC-Säulen

GE Healthcare

Data file 18-1115-23 AD BioProcess Column

BPG Columns 100, 140, 200, 300 and 450 series

BPG™ columns are glass chromatography columns designed for industrial applications which demand high standards of hygiene. The columns are constructed from component materials of the highest quality and withstand the harsh conditions used for cleaning in place of packed separation media. An overview of column characteristics is shown in Table 1. The columns are characterized by:

- Hygienic design and operation. Microbial attachment and growth is hindered through the use of calibrated precision glass, high grade electropolished stainless steel and an absence of dead pockets.
- Easy, efficient packing and running with the singlescrew adapter.
- Operating pressures matching most BioProcess™ Media.
- All polymeric materials meet the requirements for USP class VI, described in USP <88> Biological Reactivity Tests, *In Vivo*.

Fig 1. BPG column family.

7.2.1 تحكم للتنقية عن طريق الكومبيوتر

data file
BioProcess systems

Now part of GE Healthcare

BioProcess MPLC/HPLC Systems

BioProcess™ MPLC/HPLC systems comprise a family of stainless steel, liquid chromatography systems for use in process-scale applications where high pressures (20–80 bar) are required. Reliable 24 hour-a-day unattended operation contributes to cost-effective processing, all the way from feed introduction to final fractionation. BioProcess MPLC/HPLC systems simplify chromatographic procedures and offer:

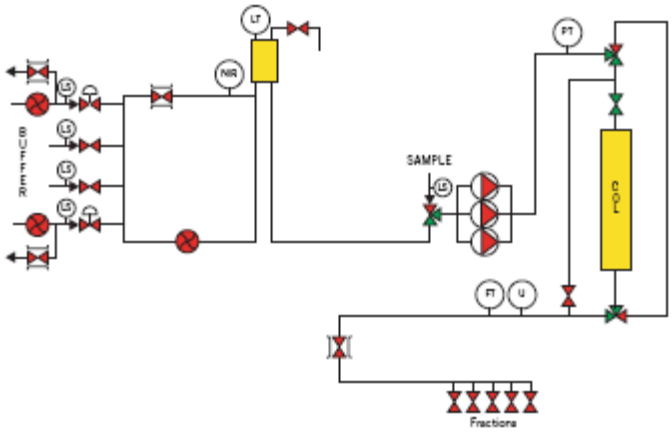
- UNICORN™ software that meets GMP requirements, including electronic signatures and records
- Precise control of gradient with feedback (Type II system only)
- Compact, modular and sanitary design
- Multi-product processing, prepared for automated CIP
- Compatible MPLC and HPLC columns

Fig 1. BioProcess MPLC and HPLC systems allow cost-saving, unattended operation in biopharmaceutical processing.

General system description

BioProcess MPLC/HPLC system – Type

The BioProcess MPLC/HPLC system – Type II is an advanced gradient system designed to blend solvents continuously. Control of the blending system is based on conductivity, NIR, or refractive index of the solvents; this results in very accurate and reproducible gradients. The inclusion of a bubble trap in the gradient system ensures that the mobile phase is free of air.



ig 3. Piping and instrumentation diagram of a BioProcess MPLC/HPLC system – Type II.

The standard configuration includes 4 inlet lines, gradient
blending loop with conductivity measurement or NIR detection,
bubble trap, pressure transmitter, flow meter after the column,
UV detector, and 5 fractionation valves. All systems are
controlled by UNICORN software.
Several additional features/components are available to ensure
that systems match specific needs. These options include:

- 2 extra inlet buffer lines
- 5 extra fractionation valves
- magnetic coupling of circulation pump
- temperature control before or after the column
- conductivity meter in gradient blending system or after the column
- pressure transmitter after the column
- pH meter before and after the column
- refractive index detector for fractionation or gradient blending system
- valve feedback
- filter module
- injection loop
- heat exchanger

UNICORN control

UNICORN control software provides a single familiar interface for both chromatography and membrane separations. It provides efficient control of the process as well as flexible method programming, extensive data evaluation and powerful reporting functionality. In addition, UNICORN is compliant with FDA 21 CFR Part 11, satisfying the regulatory requirements for electronic records and signatures.

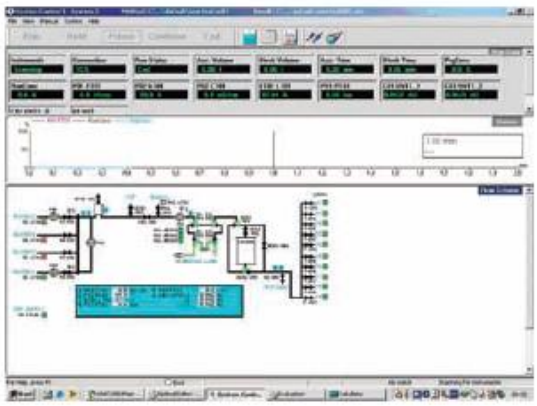


Fig 4. Same familiar interface for both chromatography and membrane system

5. BioProcess HPLC systems and HPLC columns are especially ble for purifying small biomolecules with media such as SOURCE. C system and column combinations are also available for ar applications.

7.3 مثال لطريق تنقية بروتين

Please refer to [Mourad 2003] Samir Mourad, "Purification and characterization of a Glucose-I phosohatase from *Pantoea Agglomerans*", Karlsruhe, 2003, www.aecenar.com/publications

8 تكنلوجيا اللقاح (Vaccine technology)

8.1 انواع الفاكسينات (vaccines)

تطبيق تكنلوجيا اللقاح تصنع أساس العلاج العصري ويلعب دور رئيسي في الطب البشري والطب البيطري ويشكل العنصر الوحيد للسيطره على عده أمراض معديه (infections).

اللقاح يبحث عن استمرار الية الدفاع الطبيعي من خلال جهازنا المناعة.

يحتوي اللقاح على مواد للوقايه

بعض اللقاحات تكون فعالة عندما تأخذ (تعطى) شفويا معظمها تأخذ من خلال الاهل او حسب برنامج زمني

مقتوله ميته او غير ناشطه (Attenuated, dead or inactivated bacteria)

قتل فيروس أو بكتيريا هي عملية ابعاد او تقليل خطر الاصابه بمرض عادتة يكون بالحرارة أو بمواد كميائيه

Attenuated and inactivated viral vaccines

لقاح ضد فيروس مقتول وغير فعال

تستعمل أجزاء الفيروس اللتي تنتشر في خلايه حيوانيه مثل بويضه ملقحه أو نسيج من خلايه جنين الدجاجه كلقاح

Toxoids and antigen based vaccines

لقاح اساسه الماده السامه المعالجه أو الجسم الغريب

بدايتا يتطلب تكبير التكتيريا من ثم معالجه الماده السامه التي صنعتها البكتيريا بالفرملداید

اما اللقاح الذي يعتمد على الجسم الغريب يتألف من أجزاء من الاجسام الغريبه الموجوده على مسبب المرض

The impact of genetic engineering on vaccine technology

تأثير هندسة الجينات على تقنية اللقاح

تكنولوجيا ربط ال دنا جعل من الممكن تصنيع أي بوليببتايد موجود على سطح مسبب المرض و هذا البوليببتايد بعد عزله من الجسم المصنع يمكن ان يستعمل كوحدات اللقاح

Peptide vaccine

لقاح يعتمد على ببتايد

لقاح يعتمد على صناعة بابتيد مشابه بتركيبته من الاسيد امينه الى قطعه قصيره من بوليببتايد تابع للجسم المسبب للمرض

Legionella pneumophila ATCC 33152

Phospholsipase A2

Vaccine vector

لقاح يعتمد على الفكتور يتم بادخال س دنا أو جين يرمز للمرض مستخرج من جسم مسبب لمرض داخل جسم غير مسبب للمرض ليتم اظهاره على سطح الخليه اذا ظهر يمكننا اعتماد هذه الخلايه لدعم جهاز المناعه ضد هذا المسبب

امثلة

Escherichia coli ATCC 25922

Escherichia coli ATCC 35150

Staphylococcus aureus ATCC 25923

Staphylococcus aureus

Production of Staphylococcus aureus Protein A United States Patent 4617266 *8.2 باتنت /*

Inventors:

Fahnestock, Stephen R. (Brookeville, MD)

Application Number:

06/489326

Publication Date:

10/14/1986

Filing Date:

04/28/1983

Export Citation:

Click for automatic bibliography generation

Assignee:

Genex Corporation (Gaithersburg, MD)

Primary Class:

435/69.1

Other Classes:

435/69.3, 435/69.4, 435/69.7, 435/252.31, 435/320.1, 435/839, 536/23.1

International Classes:

C12N15/09; ***C07H21/04***; ***C07K14/31***; ***C12N1/20***; ***C12N15/75***; ***C12N15/90***; ***C12P21/00***; **C12N15/09**; **C07H21/00**; **C07K14/195**; **C12N1/20**;**C12N15/74**; **C12N15/87**; **C12P21/00**; (IPC1-7): C12P21/00; C12N1/00; C12N1/20; C12N15/00

Field of Search:

435/68-71, 435/172, 435/253, 435/317, 435/172.3, 935/6, 935/11, 935/29, 935/38

View Patent Images:

Download PDF 4617266

Other References:

Kreft et al, Current Topics in Microbiology and Immunity vol. 96, pp. 1-17, (1982).
Young, Journal of General Microbiology, vol. 119, pp. 1-15 (1980).
Sven Lofdahl, et al., Gene for Staphylococcal Protein A, 10/26/82, pp. 697-701, PNAS, vol. 80.
Arne Forsgren, Significance of Protein A Production by Staphylococci, 08/04/70, pp. 672-673, Infection and Immunity, vol. 2.
John Sjoquist, et al., Localization of Protein A in the Bacteria, 05/23/72, pp. 190-194, Eur. J. Biochem, vol. 30.
Roger Lindmark, et al., Extracellular Protein A from Methicillin-Resistant Strain of Staphylococcus aureus, 10/26/76, pp. 623-628, Euro. J. Biochem, vol. 30.

Primary Examiner:

Tanenholtz, Alvin E.

Attorney, Agent or Firm:

Bernard, Rothwell & Brown

Claims:

What is claimed is:

1. A plasmid vector comprising a DNA sequence specifying Protein A; expression signals operably linked to said DNA sequence for directing expression of the Protein A DNA sequence in a Gram-positive microorganism of the species "B. subtilis"; a selectable phenotypic marker which is expressable in a Gram-positive microorganism of the species "B. subtilis"; and a region of sequence homology with a chromosome of a Gram-positive microorganism of the species "B. substilis", said region of sequence homology being capable of permitting integration of the Protein A DNA sequence into said chromosome.

2. The plasmid vector of claim 1 further comprising a selectable phenotypic marker which is expressable in E. coli and a functional E. coli replicon.

3. A method of producing Protein A comprising cultivating in a nutrient medium a transformed Gram-positive microorganism of the species "B. subtilis" transformed by a vector containing a nucleotide sequence coding for Protein A and expression signals operably linked to said DNA sequence for directing expression of Protein A in the transformed microorganism, to produce protein A; wherein the vector contains a DNA fragment homologous to a region of the chromosome of said microorganism, and the nucleotide sequence coding for Protein A is integrated into the microorganism host chromosome under recombination conditions.

4. The method of claim 3 wherein the vector is a plasmid capable of replication in E. coli.

5. The method of claim 4 wherein plasmids are prepared which contain DNA fragments homologous to different regions of the chromosome of the Gram-positive microorganism, and such plasmids are linearly integrated into their respective homologous regions of the host chromosome under recombination conditions resulting in multiple insertions into the host chromosome.

6. The method of claim 4 wherein the plasmid contains a DNA fragment homologous to sequences of the chromosomes of more than one species of Gram-positive bacteria.

7. The method of claim 4 wherein the plasmid contains more than one copy of the nucleotide sequence coding for Protein A.

8. The method of claim 4 wherein the plasmid contains more than one copy of the nucleotide sequence

coding for Protein A.

9. The method of claim 4 wherein the plasmid contains two tandem copies of the nucleotide sequence coding for Protein A.

10. A protein A-producing strain of a Gram-positive microorganism of the species "B. subtilis", wherein the chromosome of said microorganism comprises a heterologous Protein A gene under the requesting central of expression signals capable of directing expression of the Protein A gene.

11. A method of producing Protein A comprising cultivating in a nutrient medium a strain of Bacillus subtilis, having the identifying characteristics of strain GX3305 (ATCC No. 39345).

12. Bacillus subtilis strain GX3305 (ATCC No. 39345).

Description:

BACKGROUND OF THE INVENTION

Protein A is a cell wall component produced by nearly all strains of Staphylococcus aureus (see e.g. Forsgren, A., Infection and Immunity 2: 672-673 [1970]); and Sjoquist, J. et al., Eur. J. Biochem. 30: 190-194 [1972]). Protein A is useful in that it binds strongly and specifically to the Fc portion of immunoglobulin IgG from a variety of mammalian sources, including human (Kronvall, G. et al., J. Immunol. 103: 828-833 [1969]). Thus this protein has been used in diagnostic applications and has potential therapeutic value.

In most S. aureus strains, at least 70% of the Protein A produced is covalently linked to the peptidoglycan of the cell wall (Sjoquist, J. et al., Eur. J. Biochem. 30: 190-194 [1972]. The site of attachment is the C-terminal region of the Protein A molecule (Sjodahl, J., Eur. J. Biochem. 73: 343-351 [1977]). Some Protein A (15-30%) is generally excreted into the growth medium, and there are several circumstances under which the fraction of Protein A which is excreted can be increased. Some methicillin resistant strains of S. aureus excrete essentially all their Protein A (Lindmark, R. et al., Eur. J. Biochem. 74: 623-628 [1977]). Low levels of puromycin increase the amount of excreted Protein A, presumably by truncating the protein and thereby eliminating its C-terminal cell wall attachment site, and protoplasts excrete nearly all the Protein A which they synthesize (Movitz, J., Eur. J. Biochem. 68: 291-299 [1976]).

Protein sequence information is available for Protein A from S. aureus strain Cowan I (Sjodahl, J., Eur. J. Biochem. 78: 471-490 [1977]). The Cowan I strain contains approximately 2×10^5 molecules of Protein A per cell (Sjoquist, J. et al., Eur. J. Biochem. 30: 190-194 [1972]).

Protein A is synthesized in S. aureus only during exponential growth, and synthesis ceases as the culture approaches stationary phase (Movitz, J., Eur. J. Biochem. 48: 131-136 [1974]). The level of synthesis of Protein A in S. aureus is highly variable, and is strongly influenced by the growth conditions in some as yet poorly defined ways (Landwall, P., J. Applied Bact. 44: 151-158 [1978]).

The Protein A gene from S. aureus strain Cowan I has been cloned in E. coli. Lofdahl, S., et al., Proc. Natl. Acad. Sci. USA, 80, 697-701 (1983). This gene is contained in a 2.15 kilobase insert bounded by EcoRV restriction sites. The gene has been inserted into a plasmid and cloned in E. coli, where low levels of expression have been achieved. The chimeric plasmid which contains the Protein A gene has been designated "pS

."

Currently, industrial production of Protein A is carried out using mutant strains of S. aureus. A major disadvantage of using S. aureus to produce Protein A is that all available production strains are human pathogens. Although many genetic engineering experiments have been conducted using Escherichia coli, that organism is not suitable for efficient production of Protein A, since it does not export protein outside the cell. Furthermore, E. coli possesses disadvantageous pathogenic properties as well, i.e., produces endotoxins.

There thus remains a need for the production of Protein A by means which are both safe and efficient.

SUMMARY OF THE INVENTION

In accordance with the present invention, Protein A-producing Gram-positive bacteria are prepared by introduction into Gram-positive cells which do not normally produce Protein A, vectors containing the nucleotide sequence coding for Protein A and expression signals directing expression of the Protein A gene in the microorganism. Protein A can be produced by cultivating such cells in a nutrient medium under protein-producing conditions.

DETAILED DESCRIPTION OF THE INVENTION

A method of achieving high level production of Protein A in Gram-positive microorganisms without substantially inhibiting the growth of the host has been discovered. The method involves transformation of a Gram-positive microorganism by introduction therein of a vector containing the nucleotide sequence coding for Protein A. A Protein A gene may be obtained from Protein A-producing microorganisms, such as the above-mentioned strains of S. aureus. A preferred source of the gene is plasmid pS

, which has been cloned in E. coli. See Lofdahl, S., et al. (supra). The gene may advantageously be excised from that clone by digestion with endonuclease EcoRV.

The Protein A gene may contain its natural expression signals (i.e., transcriptional and translational initiation sequences), or those signals may be replaced by other expression signals recognizable by the Gram-positive host microorganism. Replacement of the natural expression signals with other recognizable Gram-positive expression signals may be accomplished using conventional methods of molecular biology. Such replacement involves cleavage of the natural expression signals from the Protein A sequence and fusion of the desired expression signals to the Protein A gene.

Construction of the Protein A-producing strains of this invention involves inserting, by recombinant DNA techniques, the Protein A gene into a plasmid vector. Such a vector may be prepared in vitro and inserted directly into the Gram-positive bacterial host by transformation techniques. The vector is preferably cloned in another organism for amplification and purification prior to transformation of the ultimate Gram-positive host cells. The microorganism used for the intermediate cloning step may be an organism in which the vector will be maintained and express selectable phenotypical properties. E. coli is the preferred microorganism for the intermediate cloning step.

When the vector is constructed in vitro and first cloned in E. coli, it advantageously contains a functional E. coli replicon as well as a phenotypic marker for E. coli. The vector also advantageously contains a phenotypic marker for the Gram-positive host microorganism. In one embodiment of the present invention, the vector also contains a functional replicon permitting autonomous replication in the Gram-positive bacteria

selected. One or more copies of the Protein A gene may be inserted into this vector, and the vector then used to transform the appropriate Gram-positive microorganism.

In preferred embodiments of this invention, the vector does not contain a replicon capable of functioning in the Gram-positive microorganism selected, but rather contains a DNA sequence homologous to a region of the chromosome of that Gram-positive microorganism. This construction permits linear integration of the vector into the host chromosome in the region of homology. The vector is again advantageously constructed in vitro and first cloned in E. coli as above; however, if desired, the gram-positive bacterial host may be transformed directly with the chimeric plasmid. Such a vector transforms the Gram-positive microorganism by recombination with the homologous region of Gram-positive host chromosome. An advantage of this method is that there is less likelihood of loss of the Protein A sequence from the host, due to negative selection favoring plasmid-free cells, and Protein-A producing strains prepared in this manner have been found to be genetically stable.

The Gram-positive host microorganisms employed in this invention are advantageously selected from non-pathogenic strains which do not normally synthesize Protein A. Although the invention will be described in detail with regard to Bacillus subtilis, it is to be understood and will be appreciated by those skilled in the art that the invention is applicable to a variety of Gram-positive microorganisms. Particularly preferred host microorganisms are well known industrial strains of the genera, Bacillus and Streptomyces. Generally, it has been found that Protein A is produced at optimum levels during the exponential growth phase of the organisms, and production slows considerably thereafter. It has also been found that the period of Protein A synthesis can be extended using sporulation deficient (spo^-) Gram-positive hosts. When spo^- hosts are used, the resulting strains are generally genetically more stable, the level of Protein A is higher, and, because fewer proteases are produced by these cells, the Protein A product is more stable.

Transformation of the Gram-positive microorganism may be accomplished by any suitable means. A particularly preferred transformation technique for these organisms is to remove the cell wall by lysozyme digestion, followed by transformation of the resulting protoplasts. Chang, S., et al. Molec. Gen. Genet., 168, 111-115 (1979). Alternatively, cells competent for transformation can be transformed by a modification of the method of Anagnostopoulos, C., et al., J. Bacteriol., 81, 741-746 (1961), as described in Example III below.

The procedures used to clone the Protein A gene and construct Protein A-producing strains of B. subtilis described herein are, except where otherwise indicated, accomplished by using conventional techniques of molecular biology. Segments of DNA containing the sequence coding for Protein A are isolated. If the sequences contain the natural expression signals for Protein A, the segments may be inserted into an appropriate vector without further modification. If the sequences do not contain the natural expression signals, or it is desired to replace them, the existing expression signals (if present) may be enzymatically removed and a DNA sequence containing the desired expression signals may then be fused to the Protein A gene. The Protein A sequences attached to the desired expression signals may then be inserted into an appropriate vector.

Vectors appropriate for transformation of B. subtilis are generally plasmids, and are advantageously constructed in E. coli. Such vectors contain a functional E. coli replicon, a phenotypic marker for E. coli, and a phenotypic marker for B. subtilis. The vector may also contain a B. subtilis replicon, but preferably it does not and instead contains a DNA sequence homologous to a region of the B. subtilis chromosome. Insertion of the homologous DNA sequence into the vector permits recombination of the vector with the B. subtilis chromosome, where it can be maintained at a copy number of one per genomic equivalent.

One or more copies of the DNA sequences coding for Protein A and the desired expression signals are then inserted into the vector. The presence of the E. coli replicon and phenotypic marker in the vector permit its cloning and maintenance in E. coli, and allow for selection of clones containing the vector.

When an intermediate cloning step in E. coli is employed, one or more E. coli colonies which carry the Protein A-containing plasmid are grown on suitable nutrient media, and the plasmids are isolated therefrom. Cells of B. subtilis (i.e. competent cells of protoplasts) are then transformed by introduction therein of the vector and successful transformants are selected by means of the B. subtilis phenotypic marker. Vectors containing a B. subtilis replicon are capable of reproducing in the host and producing Protein A when the cells are grown under protein-producing conditions. Alternatively, vectors not containing a B. subtilis replicon but instead containing a DNA sequence homologous with the host chromosome will recombine with the host chromosome and be replicated along with the host chromosome.

Preferred plasmid vectors for the cloning procedures described herein are graphically illustrated in the drawings.

FIG. 1 depicts a vector designated pGX251, which was constructed from the E. coli vector pBR322 and the B. subtilis vector pC194. In FIG. 1, the pC194 sequence 10 containing B. subtilis replicon 30 is fused to pBR322 sequence 20 containing E. coli replicon 40. The pC194 sequence contains the CAT gene, which specifies resistance to chloramphenicol. The pBR322 sequence contains a galactokinase (galK) gene, a gene specifying ampicillin resistance (amp) and a transcription termination sequence 70 derived from bacteriophage lambda. Plasmid pGX251 contains a unique EcoRV restriction site which provides a convenient insertion site for the EcoRV fragment 60 from pS

which contains the protein A gene (pra). The most prevalent orientation of the pra gene is illustrated, but either orientation can be employed, since the pS

EcoRV segment contains the appropriate expression signals.

FIG. 2 depicts plasmid pGX284, which is the preferred vector for the practice of the present invention. In FIG. 2, pBR322 sequence 15, containing E. coli replicon 55 was fused to pC194 sequence 25 containing no replicon. Like vector pGX251, pGX284 specifies ampicillin (amp) and chloramphenicol (CAT) resistance, contains the galactokinase (galK) gene, contains the lambda transcription termination sequence and the unique EcoRV recognition sequence 65, providing an insertion site for the Protein A gene-containing segment 45. In addition, pGX284 contains a segment of B. subtilis chromosomal sequences 35. The presence of these sequences and the absence of a B. subtilis replicon permits linear integration of this vector into the chromosomes of B. subtilis transformants.

A further embodiment of the present invention involves preparation of vectors differing in their homologous chromosomal DNA sequences, but still containing one or more copies of the Protein A gene. Thus, vectors can contain sequences from different regions of the B. subtilis chromosome, or even from chromosomes of different species of Bacillus. This permits integration of the vectors into different parts of the host chromosome in the corresponding regions of homology resulting in transformants with more than one vector incorporated in the host chromosome.

Transformed B. subtilis cells are grown in a nutrient medium under protein-producing conditions resulting in the production of Protein A by the cells and the secretion of Protein A into the medium. Protein A may then be purified from the medium after removing intact cells using conventional techniques.

Those skilled in the art will recognize that, although the present disclosure describes cloning and expression of the entire Protein A gene, functional segments of that gene or fusions of the gene with other DNA segments can also be cloned and expressed in accordance with the teachings herein. Such segments and fusions are, therefore, intended to be within the scope of this invention.

The invention is further illustrated by the following examples which are not intended to be limiting. For the DNA manipulation described in this and the following examples, the restriction endonucleases and other enzymes used were purchased from New England Biolabs, Inc., Bethesda Research Laboratories, Inc., Boehringer Mannheim GmbH, and were used in the conventional manner as recommended by the manufacturer, except as noted otherwise.

EXAMPLE I

Isolation of a DNA sequence containing the Protein A gene and Promoter

Region

Plasmid pSPAI (consisting of a 7.6 kilobase pair insert of DNA derived from S. aureus strain 8325-4 in E. coli vector pBR322) at a concentration of 110 µg/ml was digested with restriction endonuclease EcoRV at 256 units/ml in a buffer ("EcoRV buffer") containing 150 mM NaCl, 6 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol for 1 hour at 37° C., then for an additional 30 min. with an additional 256 units/ml EcoRV endonuclease. A small EcoRV fragment (2.15 kb) was isolated by agarose gel electrophoresis and electroelution, and found to obtain the Protein A gene and promoter region (see Examples II and IV).

EXAMPLE II

Insertion of the 2.15 kb pair fragment into Plasmid pGX251

Plasmid pGX251 (containing an E. coli replicon derived from plasmid pBR322, a B. subtilis replicon derived from plasmid pC194, the gene for ampicillin resistance, the gene for chloramphenicol resistance and a unique EcoRV site) was linearized by restriction endonuclease digestion with EcoRV (640 units/ml) at a concentration of 40 µg/ml in EcoRV buffer for 1 hour at 37° C. Digestion was terminated by incubation for 8 minutes at 65° C. and was determined to be complete by agarose gel electrophoresis. The 2.15 kb EcoRV fragment from Example I and linearized pGX251 were ligated at a concentration of 200 µg/ml EcoRV fragment, 100 µg/ml linearized pGX251, in a buffer ("ligation buffer") containing 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 2 mM dithiothreitol, 0.5 mM ATP, and 100 µg/ml bovine serum albumin, and 4×10^5Units/ml T4 DNA ligase at 5° C. for 15 hours.

Calcium-shocked E. coli strain SK2267 (F^-, gal^-, thi^-, T_1^R, hsdR4, $recA^-$, $endA^-$, sbcB15) cells (0.2 ml), prepared as described by R. W. Davis, et al., "Advances Bacterial Genetics" Cold Spring Harbor Laboratory, N.Y. (1980) were transformed with the ligation mixture containing 0.2 µg linearized pGX251 and 0.4 µg of the 2.15 kb Eco RV fragment. Colonies were selected on standard L-broth plates containing 50 µg/ml ampicillin. An ampicillin resistant transformant designated strain GX3311 produced approximately 1 µg/A_{600} unit of Protein A, determined by the method of Lofdahl, et al. (supra). The plasmid carried by this strain, designated pgX2901, consisted of a single copy of the 2.15 kb Eco RV fragment in pGX251.

EXAMPLE III

B. subtilis competent cell transformation by pGX251 containing Protein A

gene and Protein A production therewith

Competent cells of B. subtilis, strain BR151 (Lovett, P. S., et al., J. Bacteriol., 127, 817-828 (1976)) were transformed with 0.3 μg/ml plasmid pGX251 containing the protein A gene. To prepare competent cells, B. subtilis strain BR151 was grown overnight at 37° C. on tryptose blood agar base (Difco). Cells were resuspended in 10 ml SPI medium supplemented with 50 μg/ml each of lysine, tryptophan, and methionine to give a reading of 50-70 on a Klett-Summerson colorimeter equipped with a green filter (Klett Mfg. Co., New York). SPI medium consists of 1.4% K_2HPO_4, 0.6% KH_2PO_4, 0.2% $(NH_4)_2SO_4$, 0.1% sodium citrate□$2H_2O$, 0.5% glucose, 0.1% yeast extract (Difco), 0.02% Bacto-Cusamino acids (Difco), and 0.02% $MgSO_4$□$7H_2O$. The cultures were incubated at 37° on a rotary shaker (200-250 rpm) for 3-41/2 hours until logarithmic growth ceased and the cells entered early stationary phase. The cells were then diluted 10 fold into the same medium supplemented with 0.5 mM $CaCl_2$. Incubation was continued for 90 min. The cells were then centrifuged for 5 min. at room temperature, and resuspended in 1/10 volume of spent medium. 1 ml aliquots of the cell suspensions were frozen in liquid nitrogen and stored at -80° C. for use.

For transformation, the frozen competent cells were thawed quickly at 37° and diluted with an equal volume of SPII medium supplemented as above with amino acids. SPII is the same as SPI except that the concentration of $MgSO_4$ is increased to 0.04% and 2 mM ethyleneglycol-bis-(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) is added. Cells (0.5 ml) are mixed with 0.1 to 5 μg of DNA in 13×100 mm glass tubes. The cell suspensions are rotated at 37° C. for 30 min. Penassay broth (Difco) (1-2 ml) is then added and incubation continued for 60 min. at 37° C. Cells are then recovered by centrifugation, resuspended in 0-2 ml Penassay broth, and plated on LB agar plates containing 5-10 μg/ml chloramphenicol.

Successful transformants were selected at 5 μg/ml chloramphenicol. The transformed B. subtilis strain was designated GX3308. This strain was shown to produce small quantitites of Protein A by the procedure of Lofdahl, et al. (supra), but lost the plasmid quickly upon culturing in a nutrient medium.

EXAMPLE IV

Insertion of 1 copy of the 2.15 kb fragment into Plasmid pGX284

Plasmid pGX284 (containing an E. coli replicon derived from plasmid pBR322, the gene for ampicillin resistance, the gene for chloramphenicol resistance, a unique EcoRV site, and an undetermined B. subtilis chromosomal sequence) was linearized by endonuclease digestion with EcoRV at a concentration of 40 μg/ml in EcoRV buffer for 1 hour at 37° C. Digestion was terminated by incubation for 8 minutes at 65° C. and was determined to be complete by agarose gel electrophoresis. The 2.15 kb EcoRV fragment from Example I and linearized pGX284 were ligated at a concentratin of 200 μg/ml EcoRV fragment, 100 μg/ml linearized pGX284 under the conditions described in Example II.

Calcium-shocked E. coli strain SK2267 cells were transformed with the ligation mixture containing 0.2 μg linearized pGX284 and 0.4 μg 2.15 kb EcoRV fragment. Colonies were isolated on standard L-broth plates containing 50 μg/ml ampicillin. An ampicillin resistant transformant designated strain GX3320 produced approximately 1 μg/A_{600} unit of Protein A. The plasmid carried by this strain, designated pGX2907 was determined to consist of a single copy of the 2.15 kb EcoRV fragment inserted into pGX284. Transformed Strain GX3320 has been deposited with the American Type Culture Collection, Rockville, Md., USA and has been designated ATCC No. 39344.

EXAMPLE V

B. subtilis protoplast transformation by pGX284 containing single copy of

Protein A gene and production of Protein A therewith

Protoplasts derived from B. subtilis strain 1S53 (spo0AΔ677) were transformed with 0.1 μg/ml plasmid pGX2907 containing a single copy of the Protein A gene. Strain 1S53 was obtained from the Bacillus Genetic Stock Center, Ohio State University, Dept. of Microbiology, 484 West 12th Ave., Columbus, Ohio 43210 USA. Successful transformants were selected at 5 μg/ml chloramphenicol. A transformant (designated strain GX3305) was found to produce approximately 50 μg/ml Protein A in the extracellular growth medium when grown in a medium containing (per liter) 33 g tryptone, 20 g Yeast extract, 7.4 g NaCl, 12 ml 3M NaOH, 8 g Na_2 HPO_4, 4 g KH_2 PO_4 for 17 hours at 37° C. GX3305 has been deposited with the American Type Culture Collection, Rockville, Md., U.S.A. and has been designated ATCC No. 39345.

EXAMPLE VI

Insertion of 2 tandem copies of the 2.15 kb fragment into Plasmid pGX284

From the same tranformation described in Example IV, an ampicillin resistant transformant was isolated (designated strain GX3202-2) which was determined by restriction endonuclease digest anlaysis to carry a plasmid (designated pGX2907-2) in which two tandem copies of the 2.15 kb EcoRV fragment had been inserted into pGX284.

EXAMPLE VII

B. subtilis transformation by pGX284 containing two tandem copies of

Protein A gene and production of Protein A therewith

Competent cells of B. subtilis strain BR151 were transformed with 0.3 μg/ml plasmid pGX2907-2, containing two tandem copies of the Protein A gene. Successful transformants were selected at 10 μg/ml chloramphenicol. One transformant designated strain GX3302-2 was grown in a standard fermenter (8L) containing 2×L Broth for 7 hours. The final yield of Protein A was 47 mg/l in the extracellular growth medium as determined by IgG binding activity by a competitive ELISA procedure as described by Lofdahl et al. (supra).

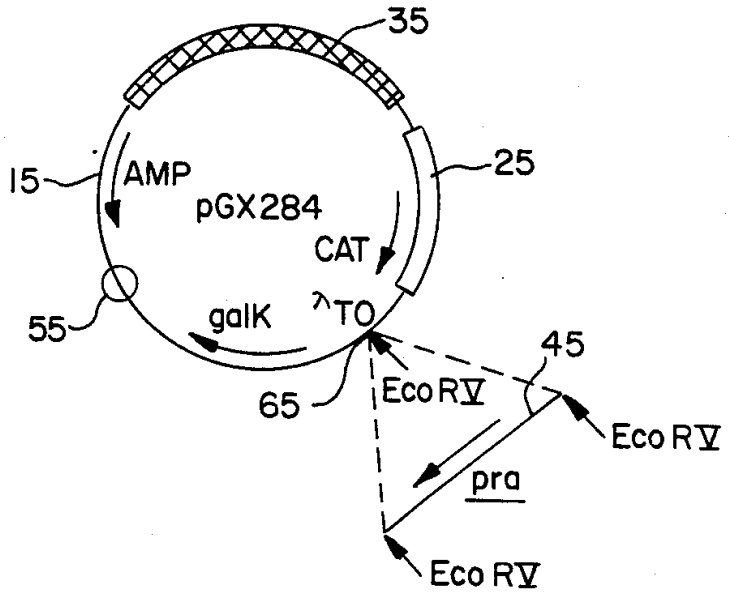


Fig. 1

FIG. 1.

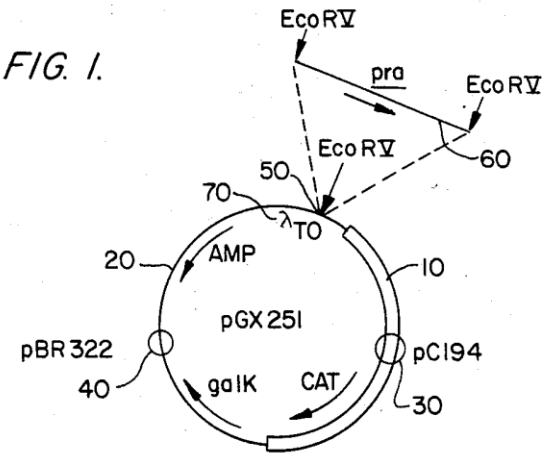
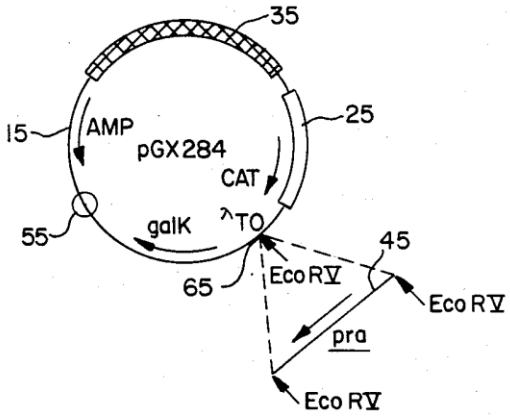


FIG. 2.



8.3 مراجع بخصوص تنقية بوروتين أ (Protein A)

Process Biochemistry Volume 33, Issue 3, March 1998, Pages 263–266

Article Title: Purification of protein A from Staphylococcus aureus by affinity chromatography using crosslinked macroporous glycidyl polymer.

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Ambedkar, S.S.

Naik, S.R.

Found In: Process biochemistry (Barking, London, England) Process biochemistry. Process biochem. 1998. v. 33 (3) p. 263-266.

Note(s): Includes references

NAL Subject(s): bacterial proteins
extraction
purity
rapid methods

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(Received 13 February 1997; revised version received 29 April 1997; accepted 6 May 1997)

Abstract

A new process for the purification of protein A from *Staphylococcus aureus* has been developed achieving purity of more than 98% with a recovery of 30%. The process involves extraction employing a specific buffer containing Toluene–Triton X-100–EDTA and subsequent purification using macroporous glycidyl copolymer. The process has been successfully used to purify protein A of both extracellular and intracellular origin. The process is simple, rapid, inexpensive and efficient when compared to many other reported processes. © 1998 Elsevier Science Ltd

Keywords: nickel removal, *staphylococcus zureus*, purification, affinity chromatography.

Introduction

Protein A is produced intracellularly or extracellularly [1] by *Staphylococcus aureus*. It is covalently linked to the cell wall and binds specifically to the Fc portion of IgG [2].

Protein A is used in different immunological tests [3] and also in the affinity purification of monoclonal antibodies. Its major therapeutic application is the removal of IgG from plasma in the treatment of certain types of cancer [4] and in the induction of endogenous interferon [5]. For therapeutic application protein A should be in a highly purified form and completely free from associated toxins.

Protein A is generally purified by gel filtration [6–8] or affinity purification [9] using an immobilized form of IgG. The matrices for IgG immobilization so far reported are: Sepharose 4B, Sepharose 6B, DEAE–Sephadex and DEAE–cellulose [10]. Application of these matrices in the purification process suffers from

There are very few reports available in the literature and no data that document the influence of process conditions on the productivity and/or economics of purification steps.

In the present communication we used glycidyl methacrylate ethylene glycol dimethacrylate, a synthetic polymer as an IgG ligand binder/coupling agent and developed a simple, rapid, reproducible and single step process for purification of protein A from *S. aureus* cells. The developed process is feasible for large scale purification of both extracellular and intracellular protein A.

Materials and methods

Chemicals

All media constituents (e.g. peptone, beef extract, glucose) were purchased from Himedia (Bombay, India). Protein A was obtained from the Sigma

one or more of the following disadvantages when compared with the multistep procedure of gel filtration; the use of hazardous chemicals to activate the matrix (e.g. cyanogen bromide), high cost and numerous operational limitations.

*To whom correspondence should be addressed.

Chemical Co. (St. Louis, MO, USA) penicillinase (ELISA grade) and polymer matrix glycidyl methacrylate ethylene glycol dimethacrylate (GMEGDMA) are the products of R & D Division, Hindustan Antibiotics Ltd (Pune, India). Other chemicals used were of analytical grade and procured from local suppliers.

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Volume 83, Issue 2, 15 November 1977, Pages 329–331

Purification of protein a from *Staphylococcus aureus* by affinity chromatography on polyacrylamide activated with thiophosgene

A.Kalyan Rao, B. Swaminathan, John C. Ayres, Joseph Mendicino

8.4 الكشف عن تركيز البروتين أ (Protein A concentration)

Biotechnology Techniques

October 1996, Volume 10, Issue 10, pp 779-782

Protein A quantitation by competitive ELISA using penicillinase

N. Muniasamy, G. M. Bhopale, S. S. Ambedkar, S. R. Naik

Summary

A simple, sensitive and specific competitive ELISA method using penicillinase as a marker for quantitation of protein A has been developed. The sensitivity of the assay is 20 ng/ml. This method is useful for estimating the protein A concentration in fermented and extracted samples.

BIOTECHNOLOGY TECHNIQUES
Volume 10 No. 10 (October 1996) pp.779-782
Received as revised 12 August

PROTEIN A QUANTITATION BY COMPETITIVE ELISA USING PENICILLINASE

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SUMMARY : A simple, sensitive and specific competitive ELISA method using penicillinase as a marker for quantitation of protein A has been developed. The sensitivity of the assay is 20 ng/ml. This method is useful for estimating the protein A concentration in fermented and extracted samples.

INTRODUCTION : Protein A is a bacterial cell-wall protein found in some strains of Staphylococcus aureus (Bjork et al., 1972). Some methicillin-resistant strains also produce protein A extracellularly (Landwall, 1978). Protein A has potential applications in immunodiagnostics (Langone, 1980), affinity purification of monoclonal antibodies and in the extracorporeal removal of IgG from plasma (Freiburghaus et al., 1988).

Protein A is quantified according to its ability to interact with the Fc portion of IgG employing different immunological methods (Mancini et al., 1965; Laurell, 1972). However, the majority of these methods are either insensitive or time consuming or both (Landwall, 1978). Until now, ELISA methods, protein A-peroxidase (Considine et al., 1986) and protein A-alkaline phosphatase (Lofdahl et al., 1983) conjugates have been used. However, these ELISA methods have disadvantages: the need for high-cost enzymes, limited availability of reagents and usage of carcinogenic chemicals (Harinath and Cheimaraj, 1992). To overcome these problems, we report here a simple, specific, inexpensive and sensitive ELISA (Competitive) for quantitation of protein A using pencillinase as an enzyme marker.

MATERIALS AND METHODS :

Preparation and Purification of Protein A-Penicillinase Conjugate :

Protein A-penicillinase conjugate was prepared by the method of Avrameas (1969). To prepare the conjugate, 2.0 ml phosphate buffer (0.1M, pH 7.0) containing 100 µl 4% glutaraldehyde, 0.2 mg penicillinase (specific activity 800 IU/mg) and 0.3 mg protein A were mixed and incubated at 30° ± 2 °C for 3 h with shaking and then dialysed against phosphate buffer (0.1 M, pH 7.0) at 4°C. The dialysed conjugate was purified using Sepharose 6B column (1.2 cm x 45 cm). The fractions of 2 ml were collected using phosphate buffer (0.05 M, pH 7.0; flow rate 30 ml/h) and absorbance of fractions was measured at 280 nm (Fig.1). Protein A-penicillinase conjugate was confirmed in 18th fraction by immunodiffusion method (Forsgen & Sjoquist, 1969) and iodometric test (Joshi et al., 1984) for protein A and penicillinase respectively.

Fermentation :

Staphylococcus aureus (ATCC 2492) was grown in 15L fermenter (New Brunswick Scientific Co.) using the culture media described by Forsgren & Sjoquist (1969). The fermentation conditions for growth and biomass separation were followed as described by Sjoquist et al. (1972).

Protein A Extraction :

The extraction methods adopted for the intra-cellular protein A were: heat extraction (Forsgren

& Sjoquist, 1969), lysostaphin (Sjoquist et al., 1972) and surfactant (0.5ml of Triton X 100 in 0.01 M phosphate buffer pH 7.0 agitated for 2 h). The protein A in extracted samples was confirmed qualitatively and quantitatively by immunodiffusion method (Forsgen & Sjoquist, 1969) and ELISA respectively.

Protein A Assay :

Each well of a microtitre plate was coated with 200 µl human IgG solution (2 µg/ml in 0.05 M $Na_2CO_3/NaHCO_3$ buffer, pH 9.6). The plates were incubated (12 h at 4 °C) and washed three times with saline (0.8% w/v) containing Tween 20 (0.05% w/v) solution. Protein A standards and protein A-penicillinase conjugate (1:40 dilution) were diluted in phosphate buffer saline (PBS, 0.01 M, pH 7.0) containing bovine serum albumin (BSA) 50 ng/ml (PBBS). The PBBS was prepared as described by Considine et al. (1986). The protein A standards (5-3000 ng/ml) were prepared in PBBS or in extracting reagents or in sterile growth media viz. Trypticase soy broth, casein hydrolysate, yeast extract. The afore mentioned media are commonly used for extracellular protein A production (Landwall, 1978). In each well of the microtitre plate 100 µl conjugate was added and then followed by 100 µl standard protein A or test samples. The contents in the well were mixed, incubated (1 h at 37 °C) and washed as mentioned earlier. Starch-Iodine penicillin v solution (200 µl) was added to each of the well and kept at room temperature (30° ± 2°C) for 30 min. The Starch-Iodine Penicillin V solution was prepared as described by Joshi et al. (1984). The enzyme-substrate reaction was stopped by adding 50 µl H_2SO_4 (1 M) and then read at 630 nm on EIA plate reader (Model: EL 310, BIO-TEK Instruments, Inc., USA). The persistance or decolourization of the blue colour indicates the presence or absence of protein A, respectively (Ambedkar et al., 1987). The intensity of the blue colour is directly proportional to the protein A concentration. Standard curve was drawn by plotting protein A concentration on X-axis and B-Bo plotted on Y-axis, where B and Bo are the absorbance values at 620 nm of tests and control wells respectively. A straight line was obtained for 20-2000 ng/ml which was selected from the linear portion of the each standard curve by linear regression analysis using the method of least squares (Howel et al., 1963).

RESULTS AND DISCUSSION :

Microtitre plate assay was carried out using 6 replicates for different protein A concentration in PBBS as described earlier. The correlation coefficient (r^2) and residual errors were calculated (Table 1) for each of standard curve, A standard graph is drawn (Fig. 2), which is suggestive of a good fit between dependent and independent variables. The correlation coefficient values are indicated even for the largest possible value of r^2 that could be observed if the relationship between the variables are correct and results are highly correlated (Waugh, 1952).

Further, the penicillinase-based ELISA was studied to validate the reproducibility of quantitation of protein A. The absorbance (B-Bo) values were determined for each of the protein A concentrations (in PBBS) and coefficient of variation was calculated (Table 2) by conducting 6 replicates of a number of protein A concentration. The values of coefficient of variation ascertain a good reproducibility, within 20-2000 ng/ml protein A concentration. Thereby suggesting penicillinase based ELISA is more sensitive than peroxidase based ELISA (Considine et al., 1986).

It was also observed that in Trypticase soy broth, casein hydrolysate yeast extract and extracting reagents did not interfere during quantitation of protein A. The values of protein A obtained from different extracts after their dilution by 100 and 200 times clearly indicate the accuracy, non interferences by the reagents and reliability of penicillinase based ELISA method (Table 3).

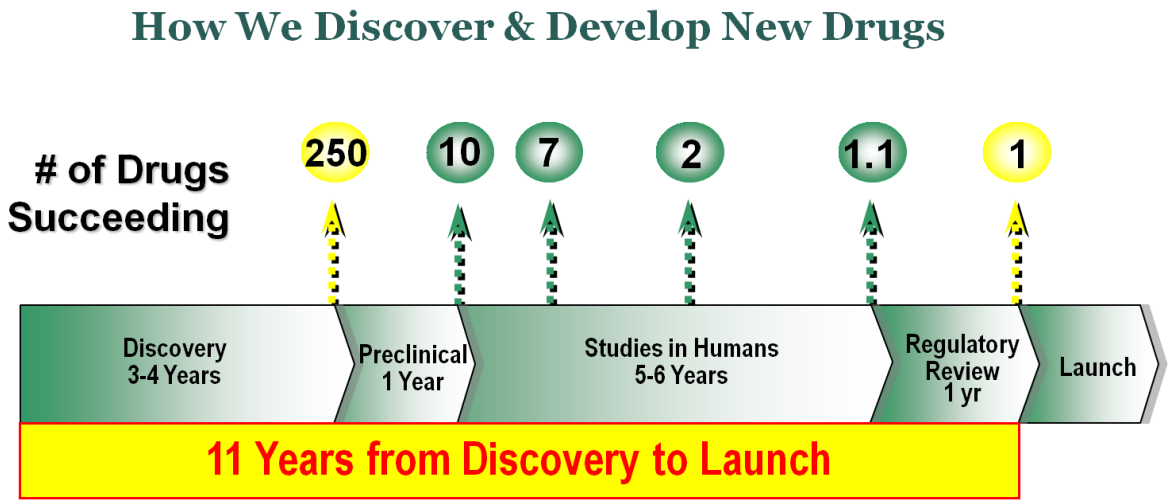
Our findings are in agreement with the earlier report of Sjoquist et al., (1972) that the efficiency of extraction of protein A from the cell of S. aureus is higher with lysostaphin as compared to heat

9 Drug development phases

9.1 بعض الاساسيات الاضافية بالنسبة للبيورياكتور و فحص دواء جديد بالحيوانات

9.1.1 Project Overview

In the figure below, you can see the drug development process



Source: PhRMA

9.1.2 Phase I: identification of drug candidate

In our case: H5N1 subunit vaccine

9.1.3 Phase II:Preclinical Tests [40]

Before a medicament is approved, the quality, safety and efficacy of any product must be demonstrated. Demonstration of conformance to these requirements is largely attained by undertaking clinical trials. However, preliminary data, especially safety data, must be obtained prior to the drug`s administration to human volunteers. These safety data, has to be explored in preclinical pharmacological and toxicological experiments accomplished in animals. Such preclinical studies can take up to 3 years to complete, and at a cost of anywhere between US$10 million and US$30 million. On average, about 10% of potential new drugs survive preclinical tests.

Provided guidelines demonstrates the range of tests during preclinical studies.

[40]Mostly from [Walsh 2007]

9.1.3.1 Pharmacokinetics and Pharmacodynamics

Pharmacokinetics relates to the fate of a drug in the body, particularly its ADME (**A**bsorption, **D**istribution, **M**etabolism, and **E**xcretion). The results of such studies not only help to identify any toxic effects, but also point to the most appropriate method of drug administration, as well as the most likely effective dosage regime to employ. Generally, ADME are undertaken in two species, usually rats and dogs, in each case males and females. Sometimes it is necessary also using primates.

Pharmacodynamic studies deal more specifically with how the drug brings about its characteristic effects. Focus in such studies is often placed upon how a drug interacts with a cell/organ type.

9.1.4 Bioavailability and Bioequivalence

Bioavailability and bioequivalence are also usually assessed in animals. The Bioavailability of a drug which is parenteral (e.g. by injection) delivered is virtually 100%. If the drug is delivered by mouth, in most cases bioavailability is given at or near 0%.

Bioequivalence studies come into play if any change in product production/delivery systems was being contemplated. These studies would seek to identify whether such modifications still yield a product equivalent to the original one in terms of safety and efficacy. Modifications could include an altered formulation or method of administration, dosage regimes, etc.

9.1.5 Protein pharmacokinetics

A condition to make pharmacokinetic/pharmacodynamic studies is the availability of an adequate selective and sensitive assay. The assay have to describe the characteristics of the therapeutic protein in the presence of a complex soup of "contaminant" molecules, how it is given in body (e.g. tissue extracts or body fluids). The specific characteristics of those drugs can be realized either via immunoassay or bioassay.

The macromolecular structure of drugs and the fact that relatively minor structural alterations can potentially have a major influence upon bioactivity are often complicating factors. For example, an immunoassay may be blind to the oxidation of an amino acid residue, or very limited proteolytic processing, although such events can activate or decrease bioactivity. Pharmacokinetic and indeed pharmacodynamic characteristics of therapeutic proteins can be rendered complicated by a number of factors, including:

The presence of serum-binding proteins. Some biopharmaceuticals (including insulin-like growth factor (IFG)) are notable in that the blood contains proteins that specifically bind them. Such binding proteins can function naturally as transporters or activators, and binding can affect characteristics such as serum elimination rates.

Immunogenicity. Many therapeutic proteins are potentially immunogenic when administered to humans. However, human proteins can also be potentially immunogenic. Antibodies raised in this way can bind the therapeutic protein, neutralizing its activity and/or affecting its serum half-life.

Sugar profile of glycoproteins. The exact glycosylation pattern which is caused by therapeutic glycoproteins in different eukaryotic expression systems, can influence protein activity and stability in vivo.

9.1.6 Toxicity studies

Acute toxicity is usually assessed by administration of a single high dose of the test drug to rodents. Both rats and mice (male and female) are usually employed. The calculation of the LD_{50} value is outdated. Nowadays, calculation of the approximate lethal dose is sufficient. Chronic toxicity studies require large numbers of animals. Most chronic toxicity studies demand daily administration of the test drug at three different dosage levels. The highest level should ideally induce a mild but observable toxic effect, whereas the lowest level should not induce any ill effects. During this process all animals are subjected to routine clinical examination, and periodic analyses (e.g. blood, urine tests).

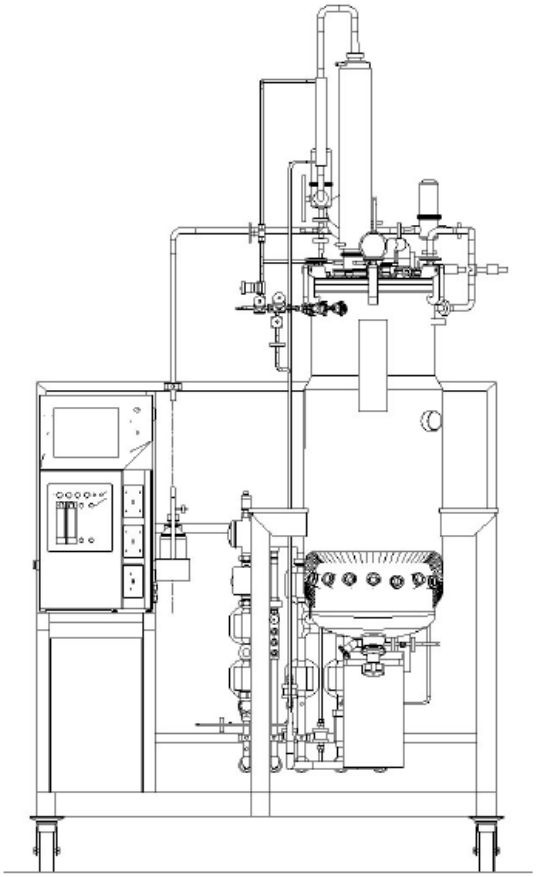
Reproductive toxicity and teratogenictiy. Fertility studies aim to assess the nature of any effect of the substance on male or female reproductive function. The drug is administered to males for at least 60 days. Females are dosed for at least 14 days before they are mated. Specific tests carried out include assessment of male spermatogenesis and female follicular development, as well as fertilization, implantation and early foetal development. A teratogen is any substance/agent that can induce foetal developmental abnormalities. Examples include alcohol, radiation and some viruses.

Mutagenicity. Mutagenicity tests aim to determine whether the proposed drug is capable of inducing DNA damage, either by inducing alterations in chromosomal structure or by promoting changes in nucleotide base sequence. These tests are usually carried out in vitro and in vivo, often using both prokaryotic and eukaryotic organisms.

For phase II: upstream – downstream without clean room

9.1.7 Phase III

Production for clinical tests (upstream – downstream – pilot site with clean room)



Bioreaktor

Pilot System 130 L

For Cell Application

10 Hepatitis B DNA vaccine technology

10.1 لقاح التهاب الكبد الفيروسي ب (HBsAg vaccine)

ويضمن هذا **اللقاح** الوقاية من عدوى التهاب الكبد الفيروسي ب .واللقاح يحتوي على واحد من البروتينات المغلفة للفيروس، وهو المستضد الموجود على السطح الخارجي لفيروس بي .(HBsAg) وهي مصنعة بواسطة خلايا فطر الخميرة، المدرج بداخلها الشفرة الوراثية للـ .HBsAg مقرر مكون من (3) جرعات، الثانية تؤخذ بعد شهر واحد على الأقل من الجرعة الأولى والثالثة تؤخذ بعد الجرعة الأولى بستة أشهر [1].بعد ذلك ينشئ جهاز المناعة أجسام مضادة ل HBsAg في الدم. وتعرف الأجسام المضادة لـ *HBsAg* ب *anti-HBsAg* هذه الأجسام المضادة وذاكرة جهاز المناعة يوفر الحصانة ضد العدوى بالتهاب الكبد الفيروسي ب [2].وأول لقاح أصبح متاح في عام 1981.

وهناك مجموعة من اللقاحات المتوفرة في السوق. في الوقت الحاضر لقاحات مصنعة بواسطة تكنولوجيا]] الهندسة الوراثية [[متوفرة، وهو ما يعني أنها تنتج عن طريق إدخال الوحدة الوراثية لالتهاب الكبد الفيروسي البائي في الخميرة المعروفة حيث يتم زرعها، وتجميعها، وتنقيتها. لا يمكن أن تحدث عدوى التهاب الكبد الفيروسي بي نتيجة أخذ اللقاح.

العلامات التجارية العروفة والمتاحة هي Engerix-B (GSK), Elovac B (Human Biologicals Institute, A division of Indian Immunologicals Limited), Genevac B (Serum Institute), Shanvac Bالخ. كل هذه التطعيمات تؤخذ في العضل.

10.2 Genetic Engineering Part: Initial inserting HBSAg into S.cerevisiae - Methods and Protocols[41]

10.2.1 Transformation von Plasmid-DNA in superkompetente

E. coli

Soll Plasmid-DNA aus *Saccharomyces cerevisiae* isoliert werden, so empfiehlt sich die Isolation mit dem „*Yeast DNA Isolation System*“ von Stratagene (siehe 2.2.7). Dabei wird eine einzelne Hefekolonie innerhalb von 10 min aufgeschlossen, kurz bei 10.000 x g in einer Tischzentrifuge (Heraeus) zentrifugiert und ein Aliquot des Überstands in superkompetente

[41] Mostly from [Braun 2001]

XL1-blue transformiert. Anschließend wird vorgegangen wie unter 2.1.7 Präparation und Reinigung von Plasmid-DNA beschrieben.

superkompetente XL1-blue Stratagene

®-**Mercaptoethanol-Stammlösung**:

®-Mercaptoethanol 1,42 M Sigma

Mg^{2+}-Lösungen:

$MgCl_2$ x 6 H_2O 1 M Merck

$MgSO_4$ x 7 H_2O 1 M Merck

beide Lösungen steril filtrieren (0,2 µm).

Glucose-Lösung:

Glucose 2 M Merck

steril filtrieren (0,2 µm).

NZY+ Broth:

Casein-Hydrolysat, säurehydrolysiert 10 g/l Roth

Hefeextrakt 5 g/l Difco

NaCl 5 g/l Roth

bei 121°C 20 min autoklavieren und vor Gebrauch pro Liter folgendes zusetzen:

je 12,5 ml der beiden Mg^{2+}-Lösungen und 10 ml Glucose-Lösung

Die bei -80°C gelagerten superkompetenten XL1-blue werden auf Eis langsam aufgetaut, vorsichtig gemischt und jeweils 75 µl in eisgekühlte 15 ml-Falconröhrchen (Falcon® 2059 polypropylene tubes) vorgelegt. Es werden jeweils 1,3 µl ®-Mercaptoethanol (Endkonz.: 25 mM) dazupipettiert und 10 min auf Eis inkubiert, wobei jeder Ansatz zu Beginn und dann alle 2 min vorsichtig durchmischt wird. Danach werden 2 µl eines Hefezelllysats (2.2.8) dazupipettiert und alles 20-30 min auf Eis inkubiert. Für die Kontrolltransformation wird anstelle des Zellysats 0,1 ng eines Kontollplasmids dazupipettiert. Die Zellen werden anschließend 40 s in einem 42°C warmen Wasserbad erhitzt, dann sofort auf Eis gestellt und 2 min inkubiert. Jedem Ansatz werden 400 µl NZY+ Broth zugesetzt und die Zellen 1 h bei 37°C und 225-250 rpm inkubiert. 5 µl der Kontrolltransformation werden auf einer LB/Amp-Agarplatte ausplattiert und über Nacht bei 37°C inkubiert. Die restlichen Transformationsansätze werden komplett auf LB-Agarplatten mit dem entsprechenden Antibiotikum ausplattiert und ebenfalls bei 37°C über Nacht inkubiert. Bei der

Kontrolltransformation sollten 100-500 Kolonien, bei den restlichen Transformationen 10-200 Kolonien auf den LB-Agarplatten wachsen.

Hybridisierung komplementärer Oligonukleotide

Für die Klonierung von DNA-Fragmenten, für die keine Matrize vorliegt, müssen koplementäre Oligonukleotide der gewünschten Sequenz konstruiert werden. Dabei werden die überhängenden Enden dieser synthetischen Oligonukleotide komplementär zu den kohäsiven Enden der zur Klonierung verwendeten Restriktionsenzyme gewählt. Auch die Klonierung kleiner DNA-Fragmente (< 100 bp), die bei 366 nm kaum sichtbar sind und deswegen nur schlecht mittels präparativer Gelelektrophorese aufgereinigt werden können, läßt sich auf diese Weise erleichtern.

Hybridisierungspuffer, pH 8,0:

Tris/HCl 10 mM Sigma
EDTA, Dinatriumsalz 1 mM Sigma
NaCl 50 mM Roth

Die synthetischen Oligonukleotide werden in Hybridisierungspuffer aufgenommen (200 pmol/µl). Je 20 µl (4 nmol) der komplementären Oligonukleotide werden zusammen in ein Eppendorfcup pipettiert, in einem Thermomixer (Eppendorf) bei 95°C für 5 min inkubiert und im ausgeschalteten Heizblock langsam auf RT abgekühlt. Die entstandenen doppelsträngigen DNA-Fragmente können anschließend sofort in ein entsprechend verdautes Plasmid ligiert (2.1.6.2) werden.

Dephosphorylierung gespaltener linearisierter DNA

Bei Klonierungen mittels Schnittstellen, deren kohäsive Enden komplementär sind (z.B. Eco RI und Mun I) sollte die Plasmid-DNA vor der Ligation dephosphoryliert werden. Ansonsten werden vorwiegend die Enden des Plasmids miteinander ligiert.

Für die Dephosphorylierung von 0,5-1 µg gespaltener DNA wird folgendes zu einem 20 µl-Restriktionsansatz[4] pipettiert:

2 µl 10 x Reaktionspuffer für Alkalische Phosphatase Boehringer
2 µl Alkalische Phosphatase (1 U/µl) Boehringer
16 µl autoklaviertes H_2O_{bidest}

Der Ansatz wird 30 min bei 37°C in einem Thermomixer (Eppendorf)

inkubiert und das gewünschte DNA-Fragment anschließend mittels präparativer Agarose-Gelelektrophorese aufgereinigt (2.1.7.3, 2.1.7.4).

10.2.2 Arbeiten mit Saccharomyces cerevisiae

10.2.2.1 Plasmide

Plasmid	Größe	Beschreibung	Referenzen
GAL4 AD-Plasmide:			
pGAD GH	7,9 kb	*LEU2*-Gen zur Selektion in Leu⁻ Hefen Amp^r zur Selektion in *E. coli*	van Aelst et al., 1993
pGAD T7	8,0 kb	*LEU2*-Gen zur Selektion in Leu⁻ Hefen Amp^r zur Selektion in *E. coli*	Clontech
GAL4 DNA-BD-Plasmide:			
pGBT 9	5,5 kb	*TRP1*-Gen zur Selektion in Trp⁻ Hefen Amp^r zur Selektion in *E. coli*	Access.: U 07646 Bartel et al., 1993
pGBK T7	7,3 kb	*TRP1*-Gen zur Selektion in Trp⁻ Hefen Kan^r zur Selektion in *E. coli*	Clontech Louret et al., 1997

Tab. 2.3: Yeast Two Hybrid-Klonierungsvektoren (Clontech).

Plasmid	Beschreibung	Referenzen
pCL 1	Wildtyp GAL4-Gen *LEU2*-Gen zur Selektion in Leu⁻ Hefestämmen Amp^r zur Selektion in *E. coli*	Fields & Song, 1989
PGAD T7-T	SV 40 *large T antigen* $_{(84\text{-}708)}$ in pGAD T7	Clontech
PGBK T7-53	Maus p53 $_{(72\text{-}390)}$ in pGBK T7	Clontech
PGBK T7-Lam	Human Lamin C $_{(66\text{-}230)}$ in pGBK T7	Clontech

Tab. 2.4: Yeast Two Hybrid-Kontrollplasmide (Clontech).

Plasmid	Größe	Beschreibung
pYES 2	5,9 kb	GAL1-Promoter, Proteinexpression Galaktose-induzierbar *URA3*-Gen zur Selektion in Ura⁻ Hefestämmen Amp^r zur Selektion in *E. coli*
pYES 3	5,9 kb	GAL1-Promoter, Proteinexpression Galaktose-induzierbar *TRP1*-Gen zur Selektion in Trp⁻ Hefestämmen Amp^r zur Selektion in *E. coli*

Tab. 2.5: Galaktose-induzierbare Expressionsvektoren (Invitrogen).

3.1.1.1. Hefestämme

Saccharomyces cerevisiae: **HF7c** (Feilotter et al., 1994)
Genotyp: *MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112, gal4-542, gal80-538,*
LYS2::GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3,
URA3::GAL4$_{17mers(x3)}$-CYC1$_{TATA}$-lacZ
Reportergene: *HIS3, lacZ*
auxotrophe Marker: *trp1, leu2, cyh$_r$2*

Saccharomyces cerevisiae: **Y187** (Harper et al., 1993)
Genotyp: *MAT⟨, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4⊗, met-, gal80⊗,*
URA3::GAL1$_{UAS}$-GAL1$_{TATA}$-lacZ
Reportergene: *lacZ*
auxotrophe Marker: *trp1, leu2*
Saccharomyces cerevisiae: **PJ69-2A** (James et al., 1996)
Genotyp: *MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4⊗, gal80⊗,*
LYS2::GAL1$_{UAS}$- GAL1$_{TATA}$-HIS3,
GAL2$_{UAS}$-GAL2$_{TATA}$-ADE2
Reportergene: *HIS3, ADE2*
auxotrophe Marker: *trp1, ura3, leu2*
Saccharomyces cerevisiae: **AH109** (James et al., 1996)
Genotyp: *MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4⊗, gal80⊗,*
LYS2::GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3,
GAL2$_{UAS}$-GAL2$_{TATA}$-ADE2,
ura3::MEL1$_{UAS}$-MEL1$_{TATA}$-lacZ
Reportergene: *HIS3, ADE2, lacZ*
auxotrophe Marker: *trp1, ura3, leu2*
Saccharomyces cerevisiae: **INV Sc1**
Genotyp: MAT⟨, his3⊗1, leu2, trp1-289, ura3-52
auxotrophe Marker: trp1, ura3, leu2, his3

3.1.1.2. Kultivierung von Saccharomyces cerevisiae,

Anlegen von Glycerolkulturen

Glycerol (autoklavieren) Merck
Kanamycin Sigma
Agar, für Platten Difco
YPD Medium Clontech/Anachem
Minimal SD Base Clontech/Anachem
Minimal SD Base / Gal / Raf Clontech/Anachem
DO-Supplemente: Clontech/Anachem
-His, -Leu, -Trp, -Ura, -His/-Trp, -Leu/-Trp, -Trp/-Ura, -Leu/-Trp/-His, -Leu/-Trp/-His/-Ade

Das Komplettmedium (YPD) wird nach Herstellerangaben in $H_2O_{bidest.}$ gelöst, mit NaOH pH 5,8 eingestellt und in einem Autoklaven mit Schnellrückkühlung (HICLAVE HV-85, NeoLab) bei 121°C 15 min autoklaviert.

Zur Herstellung von Selektionsmedium (*synthetic dropout, SD*) wird Minimal SD Base bzw. Minimal SD Base/Gal/Raf zusammen mit dem gewünschten DO Supplement nach Herstellerangaben in $H_2O_{bidest.}$ gelöst, mit NaOH pH 5,8 eingestellt und in einem Autoklaven mit Schnellrückkühlung bei 121°C 15 min autoklaviert.

Zur Herstellung von Agar-Platten wird dem Flüssigmedium 20 g/l Agar zugesetzt, **anschließend** der pH-Wert mit NaOH oder HCl auf 5,8 eingestellt und in einem Autoklaven mit Schnellrückkühlung bei 121°C 15 min autoklaviert.

Bei Bedarf kann dem Medium nach dem Autoklavieren 15 µg/ml Kanamycin (Stammlösung: 30 mg/ml in EtOH) zugesetzt werden. Dabei sollte das Medium aber schon abgekühlt sein (max. 50°C).

Die Kultivierung von S*accharomyces cerevisiae* erfolgt im allgemeinen über Nacht in Flüssigmedium bei 30°C und 220 rpm in Schikanekolben.

Zum Anlegen von **Glycerol-Kulturen** werden die Hefezellen bis zu einer OD_{600} von 0,5-0,7 (logarithmisches Wachstum) in Flüssigmedium kultiviert. Die Zellen werden bei 5.000 x g für 5-10 min pelletiert und das Pellet in demselben Volumen Flüssigmedium mit 25% Glycerin resuspendiert. Die Aliquots werden bei -80°C gelagert.

3.1.1.3. Kreuzung von Saccharomyces cerevisiae Stämmen (Mating)

Die Zellen zweier Hefestämme mit passendem *mating type* (MATa, MATα) können zu einem diploiden Hefestamm, mit den Eigenschaften beider Stämme, verschmelzen.
Von jedem der beiden vortransformierten Hefestämme wird eine 2-3 mm große Kolonie (< 2 Monate alt) gepickt und in 500 µl YPD-Medium (mit 15 µg/ml Kanamycin) in einem 1,5 ml Eppendorfcup resuspendiert. Die Zellen werden ca. 20 h bei 30°C und 200 rpm kultiviert, auf SD-Mediumplatten ausplattiert und 2-3 Tage bei 30°C inkubiert.

3.1.1.4. Transformation von Plasmid-DNA in *Saccharomyces cerevisiae*

Heringssperma-DNA 10 mg/ml Stratagene
DMSO 100 % Sigma

PEG-Stammlösung:
PEG 3350 50 % Sigma
bei 121°C 20 min autoklavieren.

TE-Puffer (10x), pH 7,5:
Tris/HCl 0,1 M Sigma
EDTA, Dinatriumsalz 10 mM Sigma
bei 121°C 20 min autoklavieren.

LiAc (10x), pH 7,5:
Lithiumacetat 1 M Sigma
pH-Wert einstellen und bei 121°C 20 min autoklavieren.

PEG / LiAc-Lösung: immer frisch aus den Stammlösungen ansetzen
Endkonz.: für 10 ml Lösung:
PEG 3.350 40 % 8 ml 50 % PEG
TE-Puffer (10x) 1 x 1 ml 10 x TE
LiAc 1 x 1 ml 10 x LiAc

Herstellung kompetenter Hefezellen

	Transformation Scale		
	Small	Large	Library
• YPD-Medium am Tag vor der Transformation mit 2-3 Hefekolonien (2-3 mm Durchmesser) in Erlenmeyer-Kolben mit Schikanen animpfen	50 ml	50 ml	150 ml
• 16-18 h bei 30°C und 200 rpm bis zur stationären Phase (OD_{600} > 1,5) kultivieren			
• Teil der Übernachtkultur in frisches Medium überführen → OD_{600} = 0,2-0,3	300 ml	300 ml	1 Liter
• weitere 2-4 h bei 30°C und 220 rpm kultivieren, bis zu OD_{600} = 0,4-0,8			
• Zellen 5 min bei 1.000 x g und RT pelletieren			
• Zellpellet in H_2O resuspendieren (vortexen)	25-50 ml	25-50 ml	500 ml
• Zellen 5 min bei 1.000 x g und RT zentrifugieren			
• Zellpellet in frisch hergestelltem, sterilem 1 x TE/LiAc resuspendieren	1,5 ml	1,5 ml	8 ml

Kompetente Hefezellen können einige Stunden bei RT gelagert werden.
Bei *Library-Screens* sollten sie aber sofort verwendet werden, um möglichst gute Ergebnisse zu erzielen.

Transformation von Plasmid-DNA in kompetente

Hefezellen

	Transformation Scale		
	Small	*Large*	*Library*
• sterile PEG/LiAc-Lösung herstellen	10 ml	10 ml	100 ml
• in jedem Cup vorlegen, gut mischen:			
BD-Vektor mit *bait*:	0,1 µg	20-100 µg	0,2-1,0 mg
AD-Vektor mit *Library*:	0,1 µg	10-50 µg	0,1-0,5 mg
Carrier DNA[6] (10 mg/ml):	0,1 mg	2 mg	20 mg
• zu jedem Ansatz die kompetenten Hefezellen zugeben und gut mischen (nicht vortexen)	0,1 ml	1 ml	8 ml
• PEG/LiAc-Lösung zugeben, vortexen	0,6 ml	6 ml	60 ml
• 30 min bei 30°C und 220 rpm inkubieren			
• DMSO zugeben, vorsichtig mischen	70 µl	700 µl	7 ml
• Hitzeschock: bei 42°C für 15 min inkubieren; *Large* und *Library Scale* gelegentlich mischen			
• Zellen auf Eis abkühlen			
• Zellen bei 1.000 x g und RT pelletieren	5 s	5 min	5 min
• Überstand abnehmen			
• Zellen in 1 x TE-Puffer resuspendieren	0,5 ml	1,0 ml[7] oder 10,0 ml	10 ml
• jeweils 100 µl / 200 µl auf einer Agarplatte (SD; Ø 100 mm / 150 mm) ausplattieren			

[6] Heringssperma Carrier-DNA vorher ca. 10 min bei 95 °C kochen, dann sofort auf Eis akühlen

[7] 1,0 ml bei simultaner Kotransformation, 10 ml bei sequentieller Transformation

3.1.1.5. Präparation von Plasmid-DNA aus Saccharomyces cerevisiae

„*Yeast DNA Isolation System*“ Stratagene

Eine Hefe-Kolonie (Ø 2-3 mm) wird in 20 µl Lysepuffer in einem 1,5 ml Eppendorfcup resuspendiert, 30 s in flüssigen Stickstoff getaucht und bei

37°C aufgetaut. Nach 5-minütiger Inkubation bei 95°C wird die Membranfraktion 30 s bei 14.000 x g in einer Tischzentrifuge (Heraeus) pelletiert und der Überstand in ein frisches Eppendorfcup überführt. 2 µl des Plasmid-haltigen Überstands werden wie unter 2.1.1.6 beschrieben in superkompetente *E. coli* transformiert. Zur Gewinnung einer ausreichenden Menge Plasmid-DNA wird vorgegangen wie unter 2.1.7.5.1 Präparation und Reinigung von DNA beschrieben.

3.1.1.6. Expression von Proteinen in Saccharomyces cerevisiae

Zur Expression von Proteinen in *Saccharomyces cerevisiae* sollten möglichst hoch exprimierende, induzierbare Vektoren, z.B. pYES 2, verwendet werden. Sollen Proteine exprimiert werden, die toxisch auf Hefe wirken, muss allerdings auf schwächer exprimierende Vektoren zurückgegriffen werden.
Nach Transformation des gewünschten Vektorkonstrukts werden die Hefen zunächst auf nicht induzierenden SD-Agarplatten ausplattiert. Die Hefezellen einer 50-100 ml-Übernachtkultur (SD-Induktionsmedium, z.B. SD Base Gal/Raf; pYES 2) werden nach 2.3.2 aufgeschlossen und die Proteinexpression mittels Polyacrylamid-Gelelektrophorese (2.3.6) überprüft. Anschließend werden die Hefen über Nacht im Großansatz (2-3 l) bis zu einer OD_{600} von 0,8-1,2 (je nach Hefestamm) kultiviert, nach 2.3.3 aufgeschlossen und das Protein anschließend z.B. über Nickelsäulen (2.3.4, Protein mit *HisTag*) angereinigt.

3.1.1.7. Aufschluß von Saccharomyces cerevisiae mittels alkalischer Lyse

Der Aufschluß erfolgt nach einer Vorschrift von Silve et al. (1991) durch alkalische Lyse mit anschließender Proteinfällung.

TCA	50 %	Sigma
Tris/HCl, pH 6,8	1 M	Merck
Lyselösung:		
NaOH	1,85 M	Merck
β-Mercaptoethanol	7 %	Sigma

β-Mercaptoethanol immer frisch zusetzen.

Die Hefezellen bis zur frühen log-Phase (H 1 x 10^7 Zellen/ml) in Flüssigkultur wachsen lassen und bei OD_{600} = 1,0 (in YPD) bzw. 0,8 (in SD Base) 3 ml der Zellsuspension 5 min bei 14.000 x g zentrifugieren. Das Zellpellet mit 1 ml H_2O_{bidest} waschen, in 100 µl frisch hergestellter Lyselösung resuspendieren und 10 min auf Eis inkubieren. Anschließend werden die Proteine durch Zusatz von 50% TCA und 5-minütiger Inkubation auf Eis gefällt und bei > 12.000 x g in 10 min pelletiert. Das Pellet wird durch Zugabe von 500 µl Tris-Puffer (1 M) neutralisiert (Pellet **nicht** resuspendieren). Das Pellet in 50-100 µl H_2O_{bidest} aufnehmen, evtl. beschallen, die Proteinkonzentration nach Bradford (2.3.5) bestimmen und mittels SDS-PAGE (2.3.6) und Western Blot (2.3.7) analysieren.

3.1.1.8. Aufschluß von Saccharomyces cerevisiae mittels Zymolase-Verdau

PBS, pH 7,5:

Na_2HPO_4 x 7 H_2O	7 mM	Sigma
NaH_2PO_4 x H_2O	3 mM	Sigma
NaCl	150 mM	Roth

Kaliumphosphat-Puffer, pH 7,5:

K_2HPO_4	1 M	Sigma
KH_2PO_4	1 M	Sigma

Sorbitol-Lösung:

Sorbitol	2 M	Sigma

Zymolase-Lösung:

20 mg/ml Zymolase 20T (ICN #320921) in Wasser; frisch ansetzen

Puffer A (10 x):

KH_2PO_4	350 mM	Sigma
$MgCl_2$ x 6 H_2O	65 mM	Merck

pH-Wert mit K_2HPO_4 auf 6,5 einstellen

Lösung B [pro ml]: 1,2 M Sorbitol in Puffer A

600 µl Sorbitol (2 M)

100 µl Puffer A (10x)

300 µl dest. Wasser

Lösung C [pro ml]:

100 µl Zymolase-Lösung

100 µl Kaliumphosphat-Puffer, pH 7,5 (1 M)

600 µl Sorbitol (2 M)

200 µl dest. Wasser

PBS mit Inhibitoren, pH 7,5 [pro 10 ml]:

		Stammlösung:	Endkonz.:	
Pepstatin A	3 ml	1 mg/ml (in DMSO)	0,1 mg/ml	Sigma
Leupeptin	30 µl	1 mg/ml	1 µg/ml	Sigma
Benzamidin	3 ml	1,5 M	150 mM	Sigma
Aprotinin	30 µl	2,5 mg/ml	2,5 µg/ml	Sigma
PMSF	30 µl	100 mM	0,1 mM	Sigma
EDTA	300µl	0,1 M	1 mM	Sigma

Die aufzuschließenden Hefezellen werden bei 5.000 x g 5 min abzentrifugiert und das Pellet in demselben Volumen Lösung B resuspendiert. Die Zellen werden erneut pelletiert, in demselben Volumen Lösung C resuspendiert und 1h bei 37°C inkubiert (Verdau der Zellwand Spheroplasten). Die empfindlichen Spheroplasten werden bei 3.500 x g und 4°C

5 min abzentrifugiert und anschließend in PBS mit Inhibitoren (½ des Zellvolumens) resuspendiert. Durch Beschallen (3-5 x, jeweils 20-30s) mit einem Ultraschallstab werden die Spheroplasten unter **Eiskühlung** aufgeschlossen. Die Membranfraktion wird bei 20.000 x g, 4°C in 20-30 min pelletiert.

3.1.1.9. Reinigung von Proteinen über Nickel-Säulen

Ni-NTA Superflow Qiagen

TALON 2 ml Disposable Gravity Column Clontech

Waschpuffer, pH 8,0:

100 mM NaCl Roth

20 mM Tris/HCl, pH 8,0 Merck

20 mM Imidazol Sigma

Elutionspuffer I, pH 8,0:

100 mM NaCl Roth

20 mM Tris/HCl, pH 8,0 Merck

50 mM Imidazol Sigma

Elutionspuffer II, pH 8,0:

100 mM NaCl Roth

20 mM Tris/HCl, pH 8,0 Merck

100 mM Imidazol Sigma

Elutionspuffer III, pH 8,0:

100 mM NaCl Roth

20 mM Tris/HCl, pH 8,0 Merck

500 mM Imidazol Sigma

Elutionspuffer IV, pH 8,0:

1 M Imidazol Sigma

Regenerierungspuffer, pH 5,0:

0,5 M MES Sigma

Die Säule mit 5-10 ml H_2O_{bidest} durchspülen, das Säulenmaterial vorsichtig aufschütteln, etwa 1 ml in die Säule füllen und absetzen lassen. Die befüllte Säule zweimal mit 1 ml Waschpuffer spülen, die Probe auftragen und 5 min inkubieren. Anschließend zweimal mit jeweils 1 ml Waschpuffer waschen und die Probe mit jeweils 1 ml Elutionspuffer I, II und III schrittweise von der Säule eluieren. Den Durchlauf, sämtliche Waschfraktionen und Eluate getrennt sammeln. Mit 1-2 ml Elutionspuffer IV kann vor Regenerierung der Säule der letzte Rest Protein heruntergespült werden. Dann wird sie mit 3 x 1 ml Waschpuffer gewaschen und anschließend mit 5 ml MES (20 mM) regeneriert. Die Säule kann nach Zugabe von 1-2 ml Waschpuffer bei 4°C gelagert werden.

3.1.1.10.Proteinbestimmung nach Bradford

Dieser Test wurde 1976 von Bradford entwickelt und eignet sich für Proteinbestimmungen in Lösungen, die Pufferchemikalien und reduzierende Stoffe enthalten. Für Proben, die Detergenzien wie Triton X-100 o.ä. enthalten ist dieser Test jedoch ungeeignet.

Spektrophotometer Ultrospec 3000	Pharmacia Biotech
BSA-Eichstandard	Boehringer

Bradford-Reagens (5x):

0,1 g	Coomassie Brilliant Blue G 250	Serva
50 ml	Ethanol, 50 % (v/v)	Merck
100 ml	Phosphorsäure, 85 %	Merck

mit destilliertem Wasser auf 250 ml auffüllen.

Diese Lösung muß vor Gebrauch 1:5 verdünnt und durch einen Faltenfilter filtriert werden und sollte außerdem mindestens 4 Wochen stehen.

Zu 1,0 ml Bradfordreagenz in einer Küvette werden 50 µl Probenlösung pipettiert. Das Ganze wird gut gemischt und nach 10 min wird die Absorption der Probenlösung bei 595 nm bestimmt. Die Probenlösung sollte 5 bis 50 µg Protein enthalten. Als Leerwert werden 50 µl des zur Probenverdünnung verwendeten proteinfreien Puffers anstelle der Probenlösung pipettiert. Ein Serumalbumin-Standard sollte im Bereich von 1 µg bis 70 µg aufgenommen werden.

10.3 HBsAg vaccine production

Table 1. Commercially available yeast-derived vaccines (selection)

Product	Trade name	Company	Recombinant host organism
HBV vaccine			
	AgB	Laboratorio Pablo Cassará; (LPC)	*H. polymorpha*
	Biovac-B	Wockhardt	*H. polymorpha*
	ButanNG	Instituto Butantan/ N.G. Biotecnologia	*H. polymorpha*
	Engerix B	GlaxoSmithKline	*S. cerevisiae*
	Enivac-HB	Panacea	*P. pastoris*
	Gene Vac-B	Serum Institute of India	*H. polymorpha*
	Hepavax-Gene	Green Cross Vaccine	*H. polymorpha*
	Probivac	Probimed	*H. polymorpha*
	Recombivax	Merck and Co., Inc.	*S. cerevisiae*
	Shanvac-B	Shanta	*P. pastoris*
Multivalent vaccines (Including rHBsAg)			
	Infanrixhexa HB+HIB+Polio+DTP	GlaxoSmithKline	*S. cerevisiae* (HBsAg)
	Tritanrix HB+HIB+DTP	GlaxoSmithKline	*S. cerevisiae* (HBsAg)
HPV vaccine	Gardasil	MSD	*S. cerevisiae*

10.3.1 Upstream Processing (عمليات المعالجة عند المنبع)

From http://www.biopharminternational.com/biopharm/Downstream+Processing/Recombinant-Vaccine-Production-in-Yeast/ArticlesStandard/Article/detail/485189

10.3.1.1 Recombinant Vaccine Production in Yeast (اللقاح المأشوب والمنتج في الخميرة).

Methylotrophic yeast provide balanced production of the membrane and protein components of a recombinant viral particle.

خميرة الـ Methylotrophic) تزودنا بمنتج متوازن من الغشاء والمكونات البروتينية من جزيئات المصدر الفيروسي المدروس

Product-containing cells are usually generated by a two fermenter cascade, consisting of a 5-L seed fermenter used to inoculate the 50-L main fermenter. The whole fermentation process, starting from a single vial from the working cell bank, yields a biomass of more than 10 g dry cell weight per liter in 55 hours. The production fermentation is carried out in synthetic medium feeding glycerol in the first phase, and a mixture of glycerol and methanol in the second phase.[7]

المنتج يحتوي على خلايا والتي تنتج عادة من 5-L seed) المخمرة والتي تُستخدم لتطعيم الـ -------؟

جرآء عملية التخمر والتي تحتوي على (

كل عمليات التخمر تبدا من فيروس مفرد من خزان الخلايا الفعالة لتنتج كتلة حيوية أكثر من 10 غرام خلايا جافة في الليتر الواحد خلال 55 ساعة .

الانتاج المخمر يكتمل في اصناع وسط مغذي غليسرولي في الطور الاول , ومزيج من الغليسرول والميثانول في الطور الثاني.

Remark: There should be used a one-way bioreactor for the first project period.

10.3.2 Downstream Processing (عمليات المعالجة عند المصب)

The vaccine particles are harvested and cells are disrupted by a sequence of ion exchange, ultra filtration, and gel filtration steps.[7] The purified HBsAg particles are formulated by adsorption to an aluminum hydroxide adjuvant and addition of a preservative. A single adult dose containing 10 or 20 g of rHBsAg may be administered in three single injections at 0, 1, and 6 months.

يتم جمع جزيئات اللقاح والخلايا تتمزق من خلال تعاقب عدة عمليات كـ عملية تبادل شوارد , عمليات فوق الفلترة , ومراحل فلترة باستخدام الجل .

تنقية جزيئات المستضد الموجود على السطح الخارجي لفيروس بي وتنتج من خلال عملية تسمى الادمصاص أو الامتزاز على هيدروكسيد الالمنيوم

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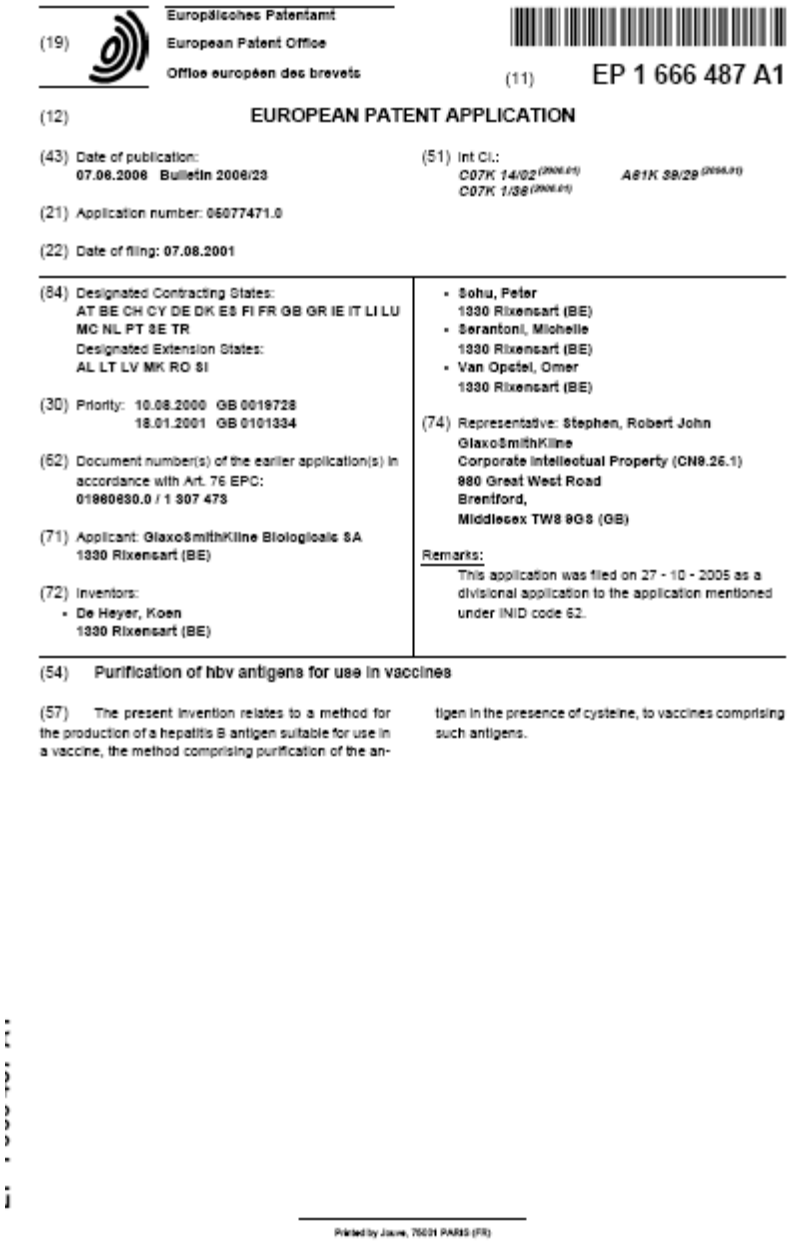
11 Downstream processing elements for Hepatitis B DNA vaccine production process

11.1 Engerix B (rHBsAg produced in S. cerevisiae)[42]



(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 666 487 A1

(12) EUROPEAN PATENT APPLICATION

(43) Date of publication:
07.06.2006 Bulletin 2006/23

(51) Int Cl.:
C07K 14/02 (2006.01) A61K 39/29 (2006.01)
C07K 1/36 (2006.01)

(21) Application number: 05077471.0

(22) Date of filing: 07.06.2001

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE TR
Designated Extension States:
AL LT LV MK RO SI

(30) Priority: 10.08.2000 GB 0019728
18.01.2001 GB 0101334

(62) Document number(s) of the earlier application(s) in accordance with Art. 76 EPC:
01960830.0 / 1 307 473

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Remarks:
This application was filed on 27 - 10 - 2005 as a divisional application to the application mentioned under INID code 62.

(54) Purification of hbv antigens for use in vaccines

(57) The present invention relates to a method for the production of a hepatitis B antigen suitable for use in a vaccine, the method comprising purification of the antigen in the presence of cysteine, to vaccines comprising such antigens.

Printed by Jouve, 75001 PARIS (FR)

The present invention relates to a method for the production of a hepatitis B antigen suitable for use in a vaccine, the method comprising purification of the antigen in the presence of cysteine, to vaccines comprising such antigens.

[42] [Patent Engerix B]

الابتكار الحالي يتعلق بطرق انتاج مستضد اللتهاب الكبد ب المناسب للاستخدام كاللقاح والطرق تتضمن تنقية المستضد خلال وجوده بسيستين ,لتلقيح يتضمن مثل هذا المستضد .

Claims:

1. A method for producing a stable, immunogenic hepatitis B vaccine without thiomersal suitable for human use, the method comprising:
2. الطريقة لإنتاج لقاح مناعي لالتهاب الكبد بدون -------؟ مناسب للاستخدام البشري والطريقة تتضمن

(a) expressing the hepatitis B surface antigen (HBsAg) as a recombinant protein in Saccharomyces cerevisiae;

يعبر عن مستضد التهاب الكبد ب (---------) ك بروتين مأشوب في (خميرة الـ)

(b) processing the yeast cells to provide a crude antigen preparation;

معالجة خلايا الخميرة لتعطي طليعة المستضد

(c) subjecting the crude antigen preparation to gel permeation chromatography, wherein the elution buffer used in the gel permeation chromatography does not contain thiomersal, thereby producing an antigen-containing eluent;

خضوع عملية تحضير طليعة المستضد للكروماتوغرافيا النفوذه للجل , والذي من خلاله محلول التصفية المستخدم في الكرماتوغرافيا النفوذه للجل لايحتوي --------- وبتلك الوسيلة يتم انتاج محلول يحتوي المستضد .

(d) subjecting the antigen-containing eluant from step (c) to ion exchange chromatography;

وبعالجة المحلول الحاوي على المستضد في المرحلة سي بعملية كروماتوغرافيا التبادل الشاردي

(e) adding cysteine to the antigen-containing eluant obtained after step (d);

اضافة السيستئين للمحلول الحاوي على المستضد والناتج عن المرحلة دي

(f) subjecting the preparation from step (e) to ultracentrifugation, thereby obtaining a purified HBsAg;

معالجة المحلول الناتج عن المرحلة السابقة لعملية الطرد المركزي , وبالتالي نحصل على مستضد التهاب الكبد النقي

(g) combining the purified HBsAg with a pharmaceutically acceptable excipient to produce a stable, immunogenic hepatitis B vaccine suitable for human use;and wherein no thiomersal is added to the resulting vaccine.

ربط المستضد النقي مع السواغ المقبول صيدلانيا لإنتاج لقاح مناعي ثابت لالتهاب الكبد نمط ب صالح للاستخدام البشري والذي يتم به عدم اضافة ---كبريتي للقاح الناتج .

2. A method according to claim 1, wherein the cysteine is added to a final concentration of between 1 and 10 mM.

الطريقة حسب الاجراء الاول , حيث تمت اضافة السيستين للتراكيز النهائي من 1 الى 10 م م

3. A method according to claim 1, wherein the cysteine is added to a final concentration of about 2 mM.

الطريقة حسب الاجراء الاول حيث السيستين قد تمت اضافته للتراكيز النهائية بحوالي 2 م م

4. A method according to claim 1, wherein the ultracentrifugation is cesium-chloride ultracentrifugation.

الطريقة حسب الاجراء الاول , حيث عملية الطرد المركزي تكون طرد مركزي بـكلوريد السيزيوم

5. A method according to claim 1 which further comprises an ion-exchange chromatography step after gel permeation (c) and before ultracentrifugation (d).

الطريقة حسب الاجراء الاول والتي تشكل لحد بعيد مرحلة كروموتوغرافيا التبادل الشاردي بعد نفوذية الجل في المرحلة سي وقبل عملة الطرد المركزي

6. A method according to claim 5, wherein the ion-exchange chromatography is anion-exchange chromatography.

الطريقة حسب الاجراء 5 حيث كروماتوغرافيا التبادل الشاردي تكون كروماتوغرافيا تبادل غير شاردي

United states

patent application publication

نشر اكتشاف مسجل

purification of HBV antigens for use vaccines

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correspondence address glaxosmithkline

عنوان المراسلة لاسم الشركة

ABSTRACT

The present invention to a method for the production of a hepatitis B antigen suitable for use in a vaccine , the method comprising purification of the antigen in the presence of cysteine to vaccines comprising such antigens.

(19) **United States**

(12) **Patent Application Publication** (10) **Pub. No.: US 2009/0123496 A1**

DE-HEYDER et al. (43) **Pub. Date: May 14, 2009**

(54) **PURIFICATION OF HBV ANTIGENS FOR USE IN VACCINES**

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(21) Appl. No.: **12/342,220**

(22) Filed: **Dec. 23, 2008**

Related U.S. Application Data

(63) Continuation of application No. 10/344,211, filed on Jul. 18, 2003, now abandoned, filed as application No. PCT/EP01/09100 on Aug. 7, 2001.

(30) **Foreign Application Priority Data**

Aug. 10, 2000 (GB) 0019728.5
Jan. 18, 2001 (GB) 0101334.1

Publication Classification

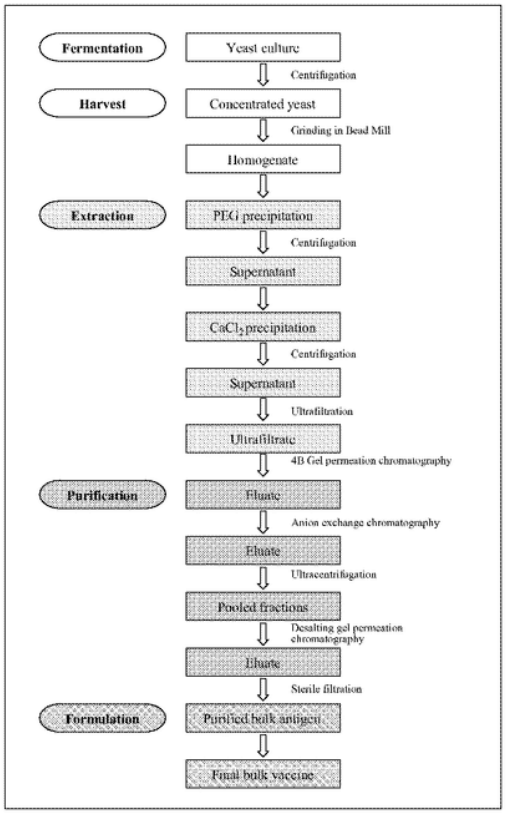
(51) **Int. Cl.**
A61K 39/29 (2006.01)

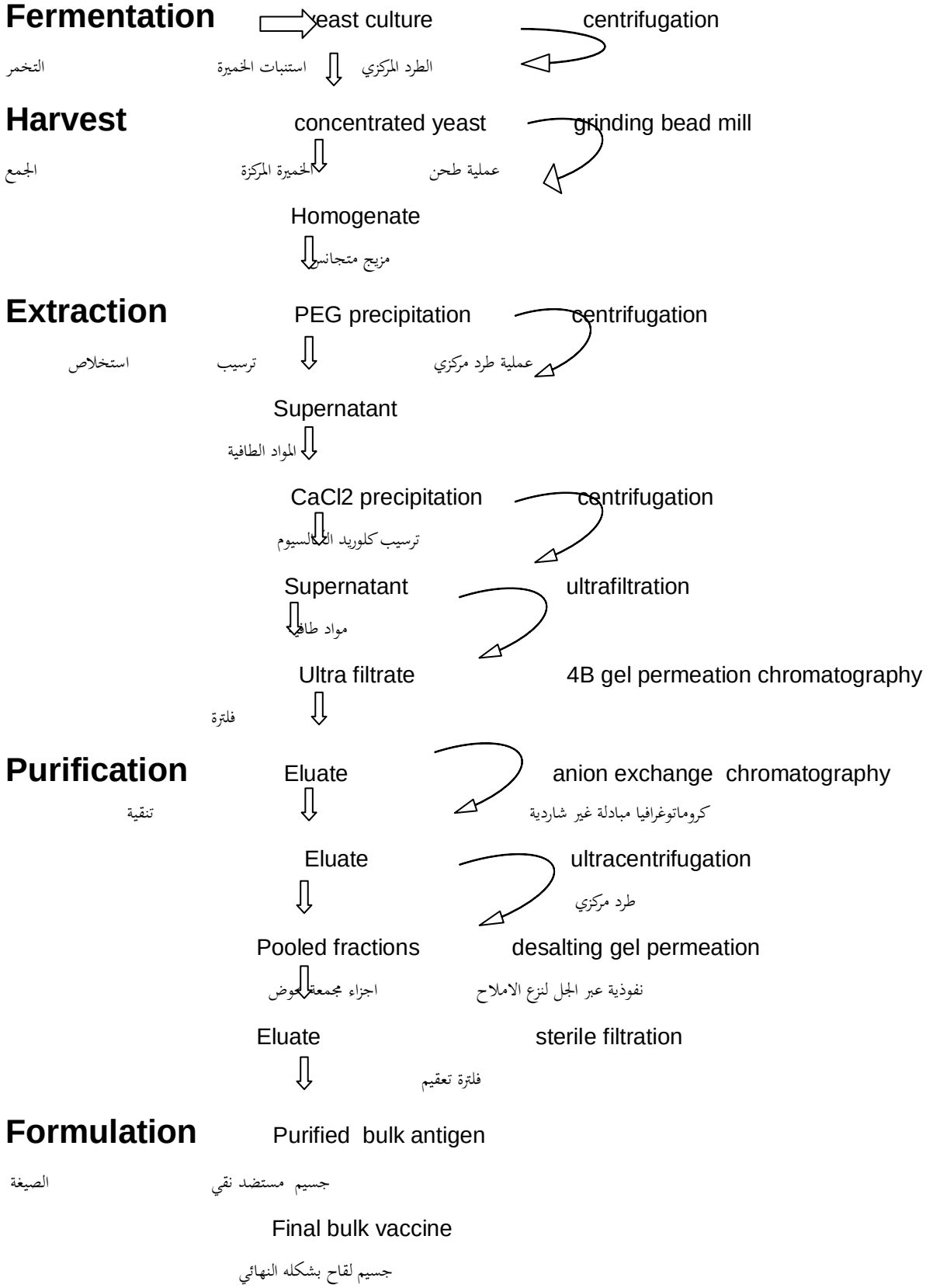
(52) **U.S. Cl.** **424/227.1**

(57) **ABSTRACT**

The present invention relates to a method for the production of a hepatitis B antigen suitable for use in a vaccine, the method comprising purification of the antigen in the presence of cysteine, to vaccines comprising such antigens.

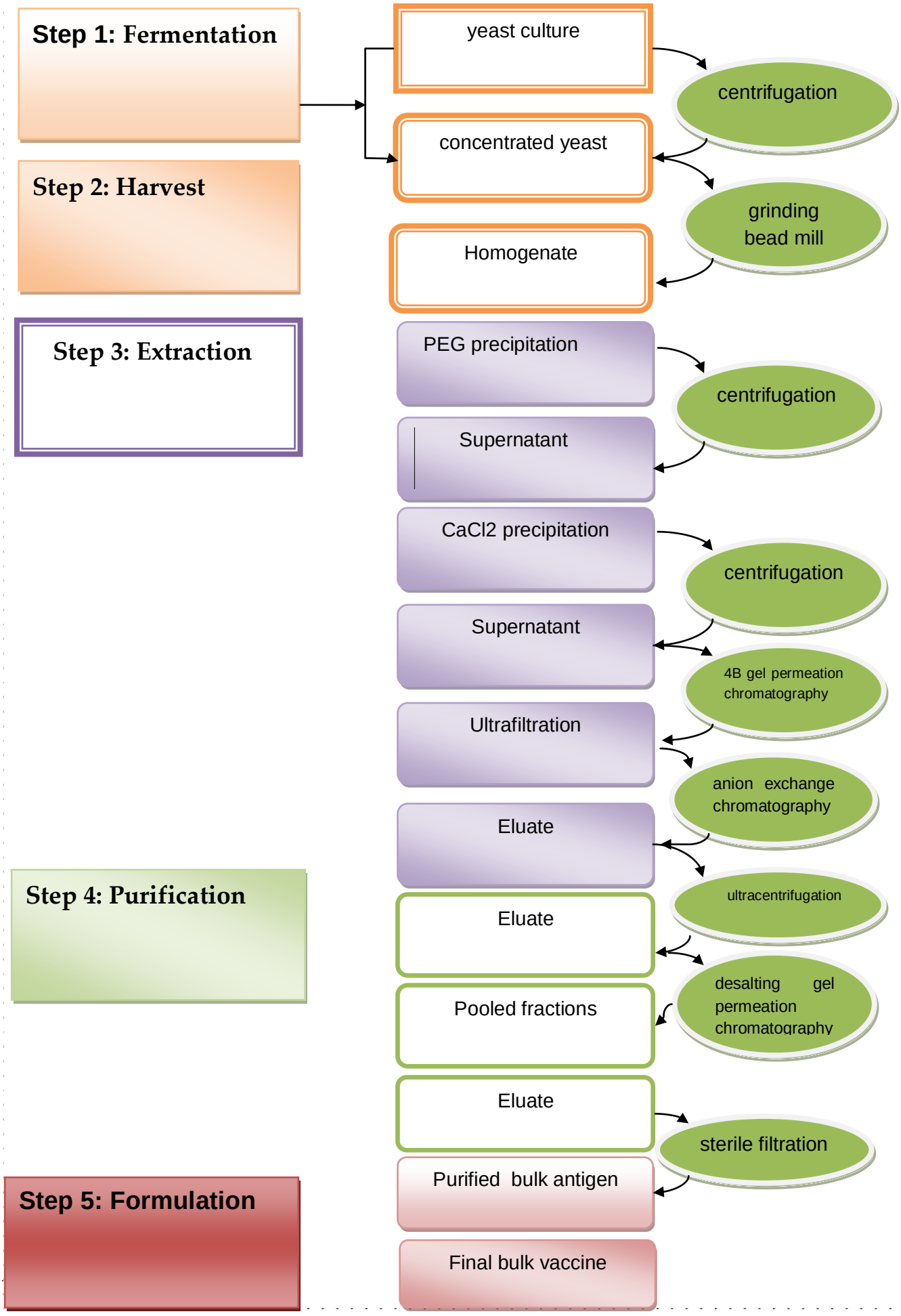
Flow diagram of the thiomersal free production process for Engereix B™




Fermentation
التخمر
yeast culture
استنبات الخميرة
centrifugation
الطرد المركزي
Harvest
الجمع
concentrated yeast
الخميرة المركزة
grinding bead mill
عملية طحن
Homogenate
مزيج متجانس
Extraction
استخلاص
PEG precipitation
ترسيب
centrifugation
عملية طرد مركزي
Supernatant
المواد الطافية
CaCl2 precipitation
ترسيب كلوريد الكالسيوم
centrifugation
Supernatant
مواد طافية
ultrafiltration
Ultra filtrate
فلترة
4B gel permeation chromatography
Purification
تنقية
Eluate
anion exchange chromatography
كروماتوغرافيا مبادلة غير شاردية
Eluate
ultracentrifugation
طرد مركزي
Pooled fractions
اجزاء مجمعة
desalting gel permeation
نفوذية عبر الجل لنزع الاملاح
Eluate
sterile filtration
فلترة تعقيم
Formulation
الصيغة
Purified bulk antigen
جسيم مستضد نقي
Final bulk vaccine
جسيم لقاح بشكله النهائي

Ba
He

Figure 1: flow diagram of the thiomersal free production process for Engerex B ™



11.1.1 Fermentation

European Patent 0199698

Produced in yeast

Burst in the precence of a non-ionic detergent, pH of the supernatant is adjusted to 6. Liquid or solid polyethylene glycol is added until supernatant is clarified.

Ultrafiltration

1.1 Outline of the production process

_[0053] Hepatitis B surface antigen may be produced by fermentation of an appropriate strain of Saccharomyces cerevisiae, for example that described in Harford et. al. (loc. cit.).

_[0054] At the end of large-_scale fermentation of the recombinant yeast strain, the cells are harvested and broken open in the presence of a mild surfactant such as Tween 20. The surface antigen is then isolated by a multistep extraction and purification procedure exactly as described above in Example 1 up to the step of the first gel permeation on Sepharose 4B.

The preparation of hepatitis B surface antigen is well documented. See for example, Harford et. al. in Develop. Biol. Standard 54, page 125 (1983), Gregg et. al. in Biotechnology, 5, page 479 (1987), EP-_A- 0 226 846, EP-_A-_0 299108 and references therein.

11.1.2 Required Devices for Downstream Processing

11.1.2.1 Thiomersal free production process

Centrifuge

Bead Mill (Grinding)

Ultrafiltration

4B Gel permeation chromatography

Anion exchange chromatography

Ultracentrifugation

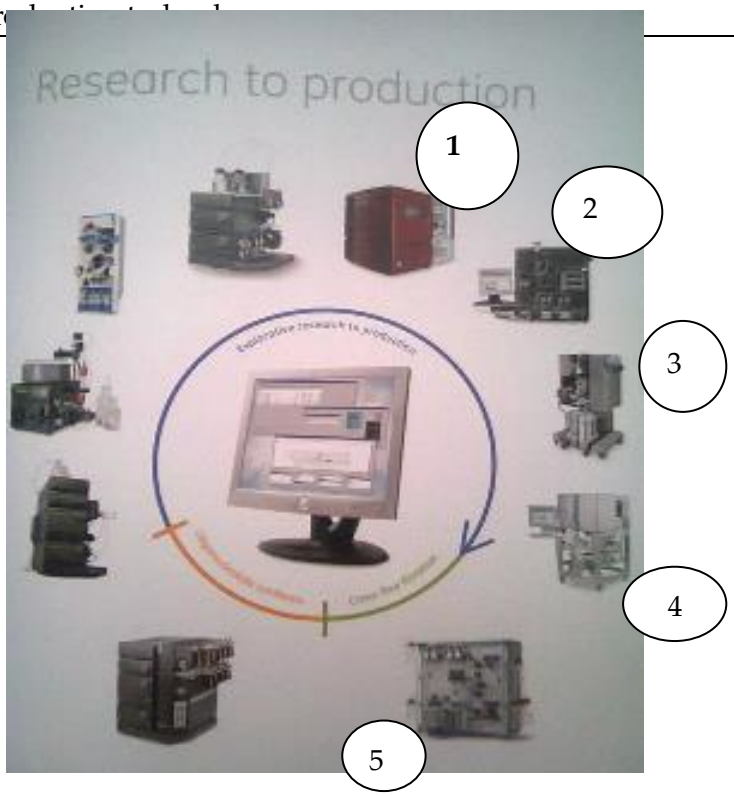
Desalting gel permeation chromatography

Sterile filtration

Requirement	Required for Step No.	Details	Details	Details
Device	4	Separation techniques	Designed to eliminate different types of yeast	
Material	4	aluminum hydroxide	adsorption	
Material	4 3	Tween 20	• Yeast strain • liberate the desired protein	Broken open
Material	4	thiomersal		
Material	4	Cysteine (amino acid)	Purification	

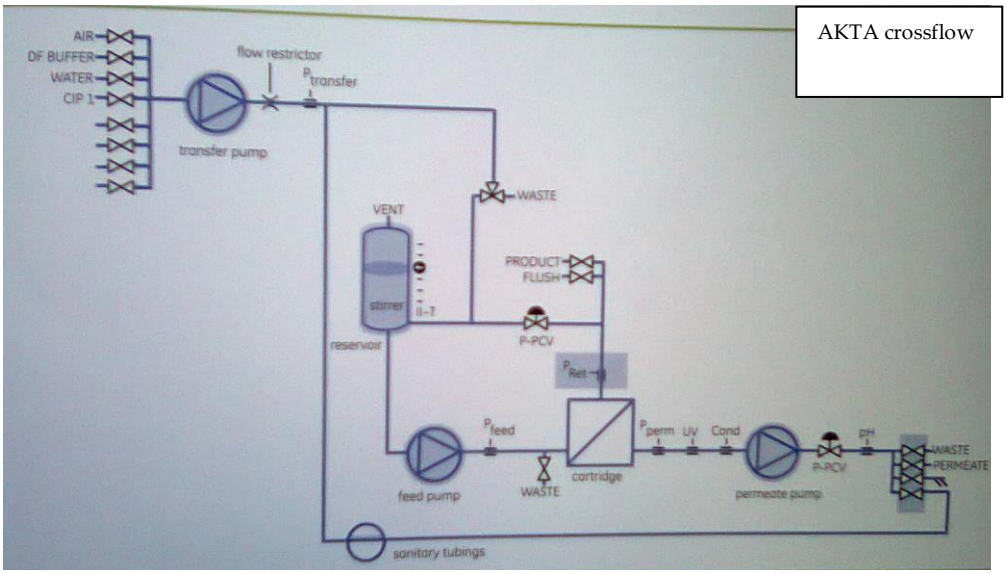
Material	5	potassium bichromate ($K_2Cr_2O_7$)		
Material	4	Sepharose 4B	extraction and purification	recombinant yeast strain (a gel permeation column)
Device	4	vapour generator		
Material	4	Mercury		
Device	2	Bead Mill (Grinding)		
Device	3	Ultrafiltration		
Device	3	4B Gel permeation chromatography		
Device	3	Anion exchange chromatography		
Device	3	Ultracentrifugation	Purification	a purified HBsAg
Device	4	Desalting gel permeation chromatography		
Device	4 1,3	Sterile filtration Centrifuge		
Material	3	CaCl2		
Material	4	the AUSZYME(kit)	Abbott Laboratories.	to assay HBsAg
Material	4	Cesium chloride CsCl	Ultracentrifugation	
Material	1	Glycerol glycerol and methanol	medium feeding medium feeding	First phase Second phase
Material	4	ELISA	immunological assay. Antibody response in mice 28 days.	
Material	3	DEAE-matrix	Ion exchange chromatography	For purification on Fast flow column.
Material	4	Sp-trisacryl ™ LS column	Supernatant of step 4 Is purified on it.	
Material	4	FORMALDEHYDE	Concentrated HBsAg eluate is treated with it	

Material	4	MINITAN ™ PELLICON ™	HBsAg solution concentrated on it.	
Material	4	SEPHACRYL 400	Purification of concentrated retentate	
Material	4	0.2 MICRON FILTER	Eluate from SEPHACRYL 400	Filtration
Device	4	Ti 15 rotor from Beckman	Zonal Centrifugation	To eliminates residual lipids, DNA and minor protein contaminants from the HBsAg preparation. It is performed by it.
Material		DNase	Treatment of HBsAg	
Material		NaCl	dialysis	Buffer solution
Material		Na pO_4	dialysis	Buffer solution
Material		DTTor 2-mercaptoethanol		
Material		TRAVENOL (kit)	Solid phase radioimmunoassay for HBsAg	
Material		ABBOTT (kit)	Known standard	
Material		CHO (kit)	Recombinant HBsAg	
Material		HEPTAVAX B R (kit)	Plasma derived HBsAg vaccine	
Material		RECOMBIVAX HB (kit)	Yeast recombinant vaccine	
Material		AUSAB ETA (kit)	To test serum for presence of anti HBsAg antibodies	
Material		WHO (kit)	Reference serum for calibration	

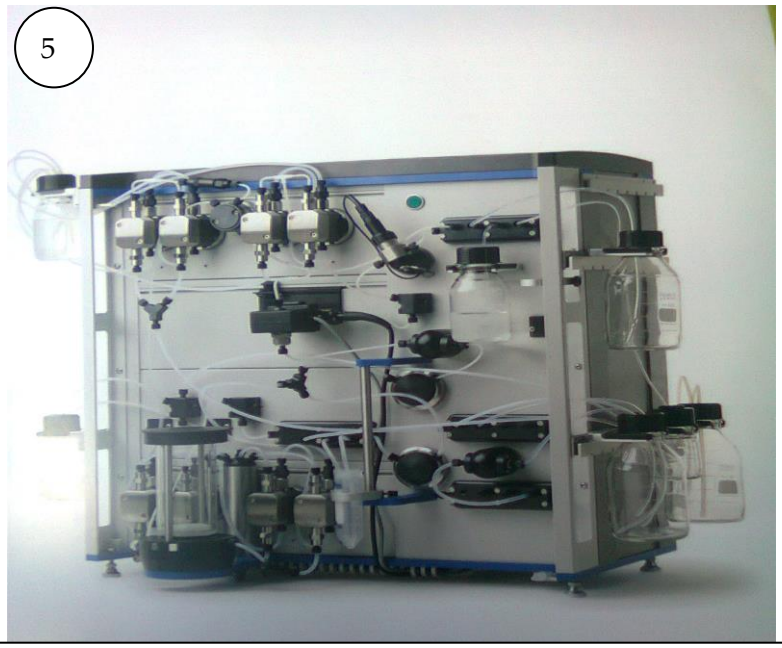


AKTA process step 4 purification (liquid chromatography)

Biopharmaceutical Manufacturing and production large scale .



AKTA crossflow



AKTA crossflow step 4 purification(automated filtration system)

AKTAmic

AKTA avant

AKTApilot

AKTA ready

GE Healthcare

AKTA

Protein purification by design

AKTA process

AKTA crossflow

3.1.1.11. Production Process of Hepatitis B Surface Antigen in the Presence of Thiomersal[43]

[0051]The Hepatitis B surface antigen (HBsAg) +of SB Biologicals hepatitis B monovalent vaccine (Engerix B®) is expressed as a recombinant protein in Saccharomyces cerevisiae (see Harford et al. loc. cit.). The 24 kD protein is produced intracellularly and accumulated in the recombinant yeast cells. At the end of the fermentation the yeast cells are harvested and disrupted in the presence of a mild surfactant such as Tween 20 to liberate the desired protein. Subsequently the cell homogenate, containing the soluble surface antigen particles, is prepurified in a series of precipitations and then concentrated via ultrafiltration.

[0052]Further purification of the recombinant antigen is performed in subsequent chromatographic separations. In a first step the crude antigen concentrate is subjected to gel permeation chromatography on Sepharose 4B medium. Thiomersal is present in the elution buffer at the 4B gel permeation chromatography step. The elution buffer has the following composition: 10 mM Tris, 5% ethylene glycol, pH 7.0, 50 mg/L thiomersal. Thiomersal is included in this buffer to control bioburden. Most of this thiomersal is removed during the subsequent purification steps including ion exchange chromatography, ultracentrifugation and desalting (gel permeation) so that purified bulk antigen preparations prepared by the original process contain about 1.2 μg and less than 2 μg of thiomersal per 20 μg of protein.

[0053] An Ion-Exchange chromatography step is performed using a DEAE-matrix and this pool is then subjected to a Cesium-gradient ultracentrifugation on 4 pre-established layers of different Cesium chloride concentrations. The antigen particles are separated from contaminating cell constituents according to their density in the gradient and eluted at the end of the centrifugation process. Cesium chloride is then removed from this pool by a second gel permeation on Sepharose gel.

[0054]When HBsAg is prepared by the process containing thiomersal in the 4B gel permeation buffer, protein concentrations of over 30 mg/ml are recovered in the pooled HBsAg containing fractions from the CsCl gradient, corresponding to an equivalent concentration of HBsAg as assayed by the AUSZYME kit from Abbott Laboratories.

[0055]The CsCl ultracentrifugation step usefully eliminates residual lipids, DNA and minor protein contaminants from the HBsAg preparation. It is performed by zonal centrifugation in a Ti 15 rotor from Beckman Instruments, Fullerton, Calif. at a speed of 30,000 rpm for about 40 to 60 hours. The sample to be purified is applied to layers of CsCl solution with final concentrations of 0.75, 1.5, 2.5 and 3.25 M CsCl. At the end of centrifugation the gradient is eluted into fractions. Fractions containing HBsAg may be identified by UV absorbance at 280 nm or by testing dilutions of the fractions with the AUSZYME kit. The HBsAg band is at a density of 1.17 to 1.23 g/cm^3.

[43] From [Patent Engerix B]

[0056]The solution containing the purified HBsAg is sterile filtered before being used to make a vaccine formulation.

[0057]Purification from the yeast cell lysate is complex as the antigen is produced intracellularly and a series of separation techniques designed to eliminate different types of (yeast) contaminants are necessary to obtain pure bulk antigen. The steps of purification are important, as the product to be purified is a lipoprotein particle containing multiple copies of the surface antigen polypeptide and this structure must be maintained throughout the purification process. It is a particularity of this process that it yields surface antigen particles which are fully immunogenic without the need for further chemical treatment to enhance immunogenicity (compare EP0135435).

[0058]The details of the production process are further described in European Patent 0199698.

11.1.3 Production and Characterization of Yeast-Derived HBsAg by a Thiomersal Free Process[44]

Production and Purification of Yeast-Derived HBsAg

1.1 Outline of the Production Process

[0059]Hepatitis B surface antigen may be produced by fermentation of an appropriate strain of Saccharomyces cerevisiae, for example that described in Harford et. al. (loc. cit.).

[0060]At the end of large-scale fermentation of the recombinant yeast strain, the cells are harvested and broken open in the presence of a mild surfactant such as Tween 20. The surface antigen is then isolated by a multistep extraction and purification procedure exactly as described above in Example 1 up to the step of the first gel permeation on Sepharose 4B.

1.2 Thiomersal-Free Purification Process

[0061]In the thiomersal free process the following two changes have been introduced compared to the process described in Example 1.

[0062]1. The elution buffer at the 4B gel permeation chromatography step no longer contains thiomersal.

[0063]2. Cysteine (2 mM final concentration) is added to the eluate pool from the anion exchange chromatography step.

[0064]It was found that omission of thiomersal from the 4B gel permeation buffer may result in precipitation of the HBsAg particles during the CsCl density gradient centrifugation step with loss of product and aggregation or clumping of the recovered antigen.

[0065]Addition of cysteine at 2 mM final concentration to the eluate pool from the preceding anion exchange chromatography step prevents precipitation and loss of antigen during CsCl density centrifugation.

[44] From [Patent Engerix B]

[0066] 2. Cysteine is a preferred substance for this treatment as it is a naturally occurring amino acid and can be removed at the subsequent desalting step on a gel permeation column using Sepharose 4BCLFF as the column matrix.

[0067] There are no other changes in the manufacturing process compared to the process described in Example 1.

[0068] The thiomersal free process yields bulk antigen of a purity and with properties comparable to antigen from the process of Example 1.

1.2a

[0069] The thiomersal added to the 4B buffer at 50 μg/ml is thought to decompose and the resulting ethyl mercury may attach covalently to free sulphydryl groups on cysteine residues of the protein. The protein contains 14 cysteine residues of which 7 are located between positions 101 and 150.

[0070]This region of the protein is believed to be located at the surface of the particle and contain the major antigenic region of HBsAg including the immunodominant a region and the recognition site for the RF1 monoclonal antibody (Waters J et al, Postgrad. Med. J., 1987:63 (Suppl. 2): 51-56. and Ashton-Rickardt and Murray J. Med. Virology, 1989:29:196). Antigen purified with thiomersal present in the 4B gel permeation buffer contains about 0.5-0.6 μg mercury at the end of the purification process. This mercury is not fully removed by simple dialysis.

[0071]In one experiment, 0.56 μg Mercury per 20 μg protein was measured on bulk antigen preparation. This preparation was dialysed for 16 hours at room temperature against 150 mM NaCl, 10 mM $NaPO_4$ buffer pH 6.9. At the end of dialysis, a concentration of 0.33 μg Hg per 20 μg protein was measured.

[0072] In contrast, dialysis in the presence of a reducing agent such as L-cysteine at 0.1 to 5.0 mg/ml, DTT at 50 mM or 2-mercaptoethanol at 0.5 M, followed by a second dialysis to remove the reducing agent, results in reduction of the mercury content of the antigen preparation to less than 0.025 μg Mercury per 20 μg protein. This is the lowest limit of detection of the method.
[0073] The mercury content was determined by absorption spectrophotometry. The antigen is diluted in a solution containing 0.01% w/v of potassium bichromate ($K_2Cr_2O_7$) and 5% v/v of nitric acid. Standard solutions are prepared with thiomersal as the mercury source. The atomic absorption of sample and standard solutions is measured after vaporisation in a vapour generator, with a mercury-specific cathode at 253.7 nm. Atomic absorption of the dilution liquid is measured as blank. The mercury content of the sample is calculated via the calibration curves obtained from the standard solutions. Results are expressed as μg of mercury per 20 μg of protein.

11.2 Production of HBSAg in Mammalian Cells

Patent from 1991

US005242812A

United States Patent [19]

Even-Chen

[11] **Patent Number: 5,242,812**

[45] **Date of Patent: Sep. 7, 1993**

[54] **METHOD FOR PRODUCTION AND PURIFICATION OF HEPATITIS B VACCINE**

[75] Inventor: **Zeev Even-Chen**, Yavneh, Israel

[73] Assignee: **Bio-Technology General Corp.**, New York, N.Y.

[21] Appl. No.: **790,485**

[22] Filed: **Nov. 12, 1991**

Related U.S. Application Data

[63] Continuation of Ser. No. 480,166, Feb. 14, 1990, abandoned, which is a continuation-in-part of Ser. No. 307,777, Feb. 7, 1989, abandoned.

[51] **Int. Cl.**[5] **C07K 3/26**; C07K 3/28; C07K 3/20; C07K 15/14

[52] **U.S. Cl.** **435/70.3**; 424/88; 424/89; 435/69.3; 530/395; 530/412; 530/414; 530/415; 530/416; 530/417; 530/806; 935/65

[58] **Field of Search** 424/88, 89; 435/70.3, 435/69.3; 530/412, 414, 416, 417, 415, 806; 935/65

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,349,539	9/1982	Wampler	424/89
4,558,011	12/1985	Brzosko et al.	435/272
4,738,926	4/1988	Hamada et al.	435/239

OTHER PUBLICATIONS

Lee et al. 1987, Journal of the Chinese Biochemical Society 16(1): 7–14.

Molnar-Kimber et al. 1988, J. Virology 62(2): 407–416.

Zwerner et al. 1979, Methods in Enzymology, vol. 58: 221–229.

Primary Examiner—Keith C. Furman

Attorney, Agent, or Firm—John P. White

[57] **ABSTRACT**

Processes are provided for producing purified, hepatitis B surface antigen particles in mammalian cells which comprise culturing mammalian cells which produce the particles in a culture medium supplemented with a serum free of high molecular weight contaminant proteins and recovering the purified, hepatitis B surface antigen particles.

Removal of molecules having a molecular weight greater than about 3×10^5 daltons by prefractionation, for example, allows cells to be grown in culture media containing high levels of fetal calf serum, removes high molecular weight contaminant proteins which may be inhibitory to cell growth and simplifies purification of HBsAg since high molecular weight contaminant proteins are the major contaminants removed by purification processes.

43 Claims, 10 Drawing Sheets

PRODUCTION AND PURIFICATION SCHEME OF HBsAG PARTICLES

STEP 1 PRODUCTION OF HBsAG PARTICLES IN TISSUE CULTURE SYSTEM USING FCS - SUPPLEMENTED CULTURE MEDIA THAT WAS PREFRACTIONATED ON A PELLICON™ 300,000 MW CUT OFF MEMBRANE - ONLY FILTRATE (PROTEINS< 300,000 MW) IS ADDED TO CELL CULTURE. RETENTATE ON THE MEMBRANE (PROTEINS> 300,000 MW) IS DISCARDED.

↓

STEP 2 COLLECT CULTURE MEDIA DAILY, POOL AND PURIFY BY FIRST CLARIFYING CRUDE MEDIUM CONTAINING HBsAG PARTICLES ON A PELLICON™ 0.22 MICRON MEMBRANE - ONLY FILTRATE IS PURIFIED FURTHER. RETENTATE (CELLS AND CELL DEBRIS) ON MEMBRANE IS DISCARDED.

↓

STEP 3 CONCENTRATION OF HBsAG PARTICLES FROM CLARIFIED CRUDE MEDIUM (FILTRATE OF STEP 2) AND DIALYSIS AGAINST PBS ON A PELLICON™ 300,000 MW CUT OFF MEMBRANE - ONLY RETENTATE IS PURIFIED FURTHER (PROTEINS ABOVE 300,000 MW). FILTRATE IS DISCARDED (PROTEINS BELOW 300,000 MW). RETENTATE CONCENTRATED FURTHER ON MINITAN™ 300,000 CUT-OFF MEMBRANE.

↓

STEP 4 RETENTATE OF STEP 3 IS PURIFIED BY GEL FILTRATION I ON A SEPHACRYL S-400™ COLUMN. FOLLOWED BY CONCENTRATION OF ELUTED HBsAG PEAK FRACTIONS ON MINITAN™ (300,000 MW CUT OFF MEMBRANE).

↓

STEP 5 CONCENTRATED HBsAG ELUATE OF STEP 4 PURIFIED FURTHER BY GEL FILTRATION II ON SEPHACRYL S-400™ COLUMN, FOLLOWED BY CONCENTRATION OF ELUTED HBsAG PEAK FRACTIONS ON MINITAN™ (300,000 MW CUT OFF MEMBRANE).

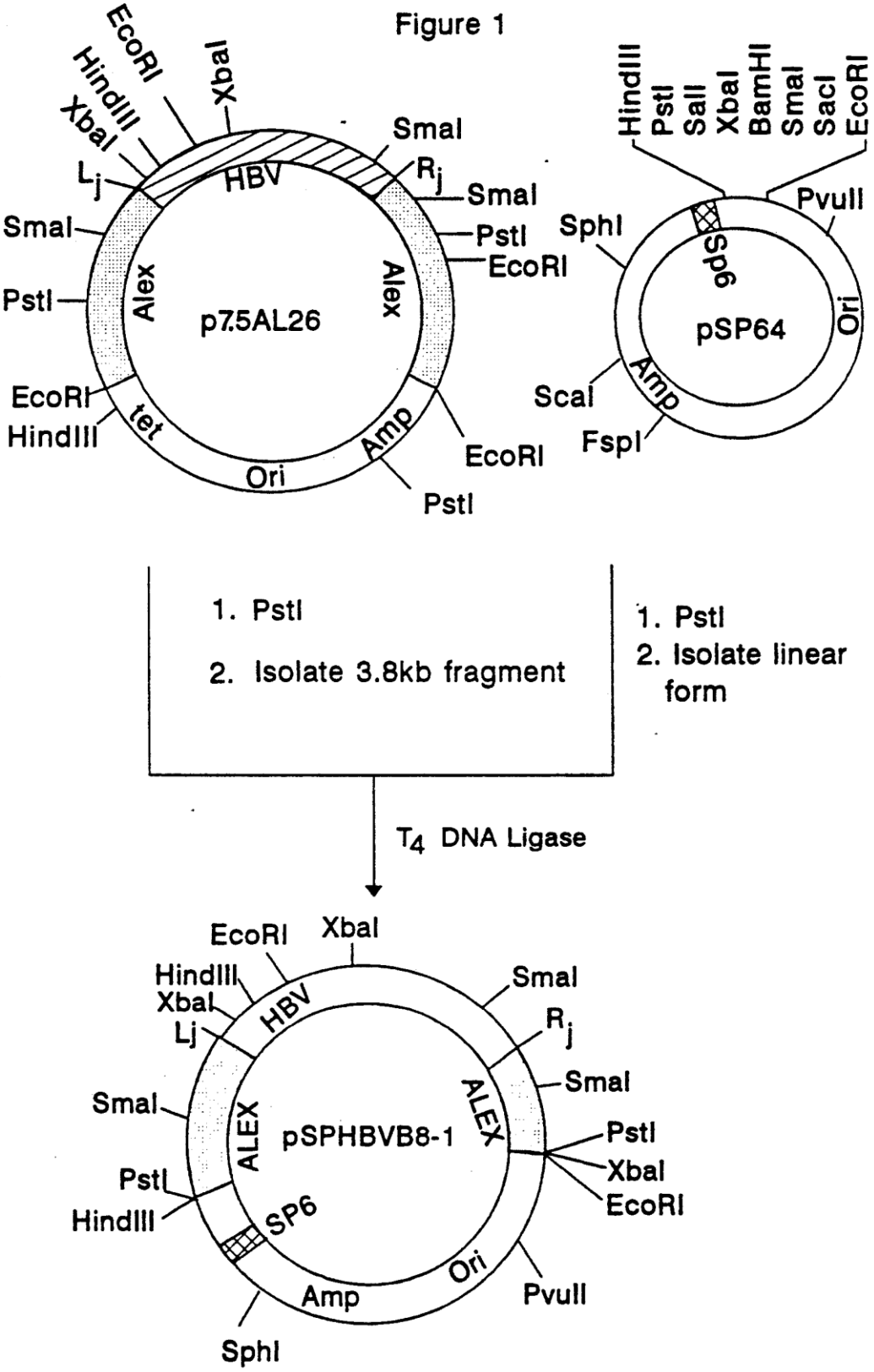
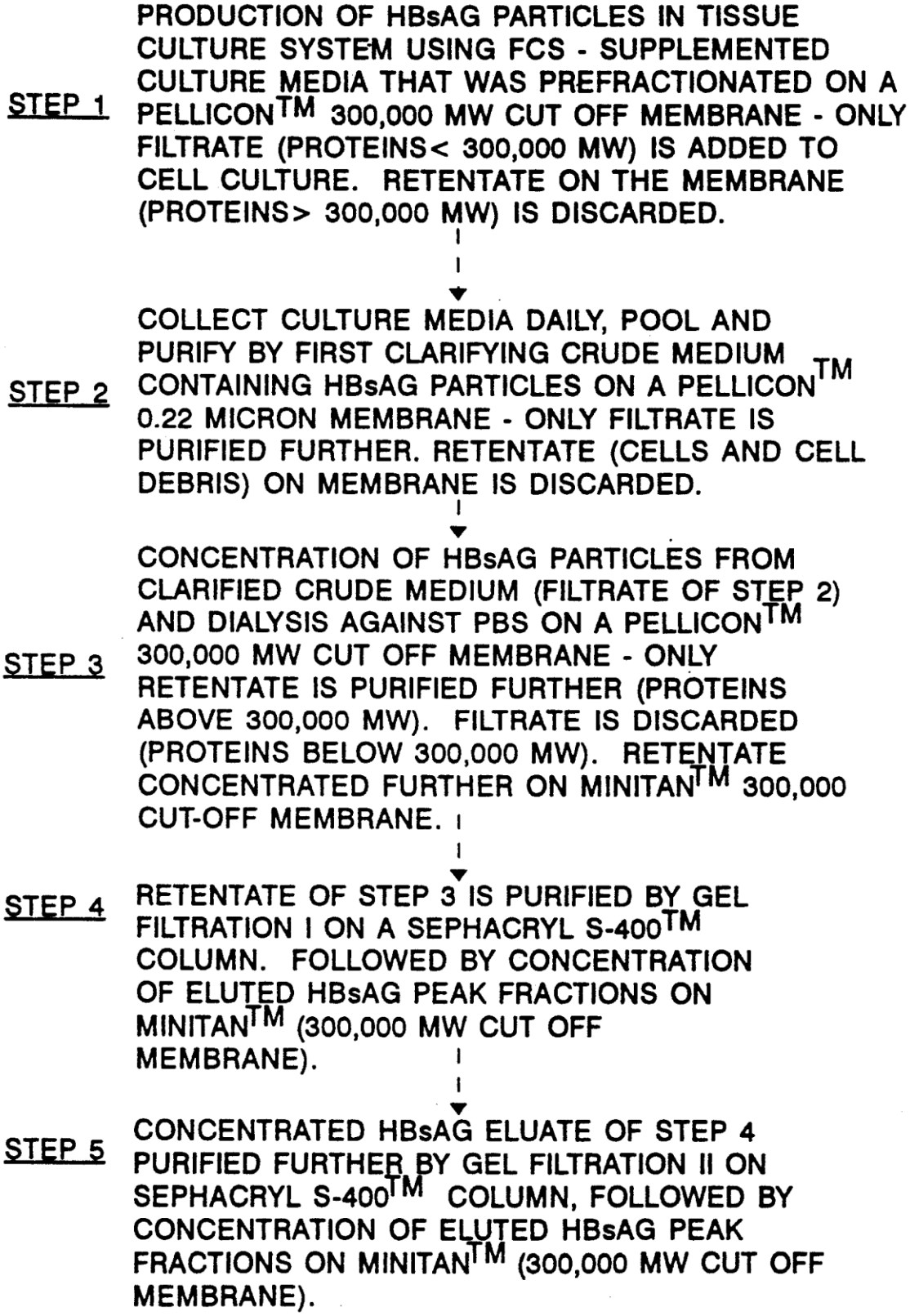


Figure 1
EcoRI
HindIII
Xbal
Xbal
Lj
HBV
SmaI
Rj
SmaI
PstI
EcoRI
SmaI
PstI
Alex
p7.5AL26
Alex
EcoRI
HindIII
tet
Amp
Ori
EcoRI
PstI
HindIII
PstI
SalI
Xbal
BamHI
SmaI
SacI
EcoRI
PvuII
SphI
SP6
pSP64
Ori
ScaI
Amp
FspI
1. PstI
2. Isolate 3.8kb fragment
1. PstI
2. Isolate linear form
T4 DNA Ligase
EcoRI
Xbal
HindIII
Xbal
HBV
SmaI
Rj
Lj
SmaI
SmaI
ALEX
pSPHBVB8-1
ALEX
PstI
Xbal
EcoRI
PstI
HindIII
SP6
Amp
Ori
PvuII
SphI

Figure 4

PRODUCTION AND PURIFICATION SCHEME OF HBsAG PARTICLES



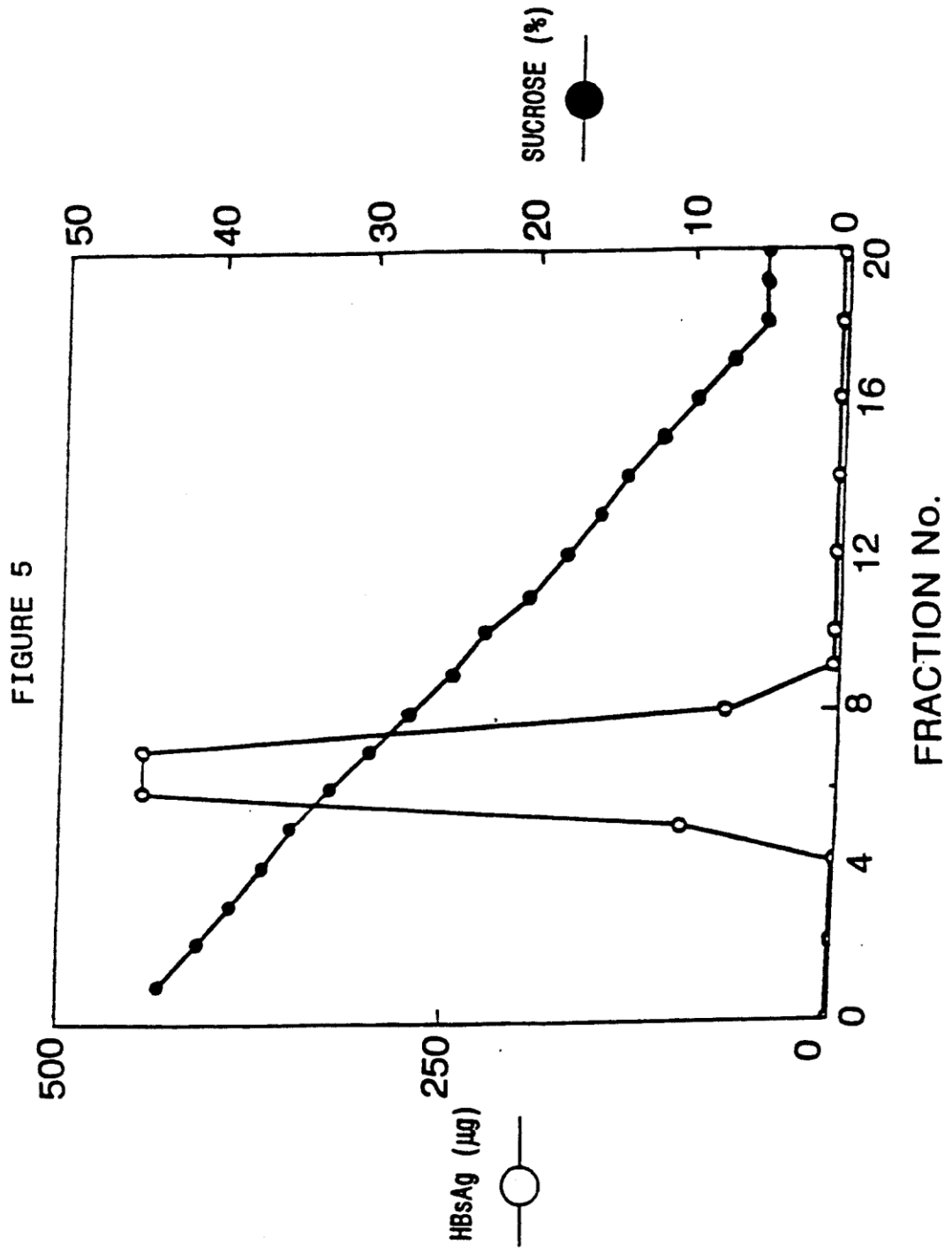
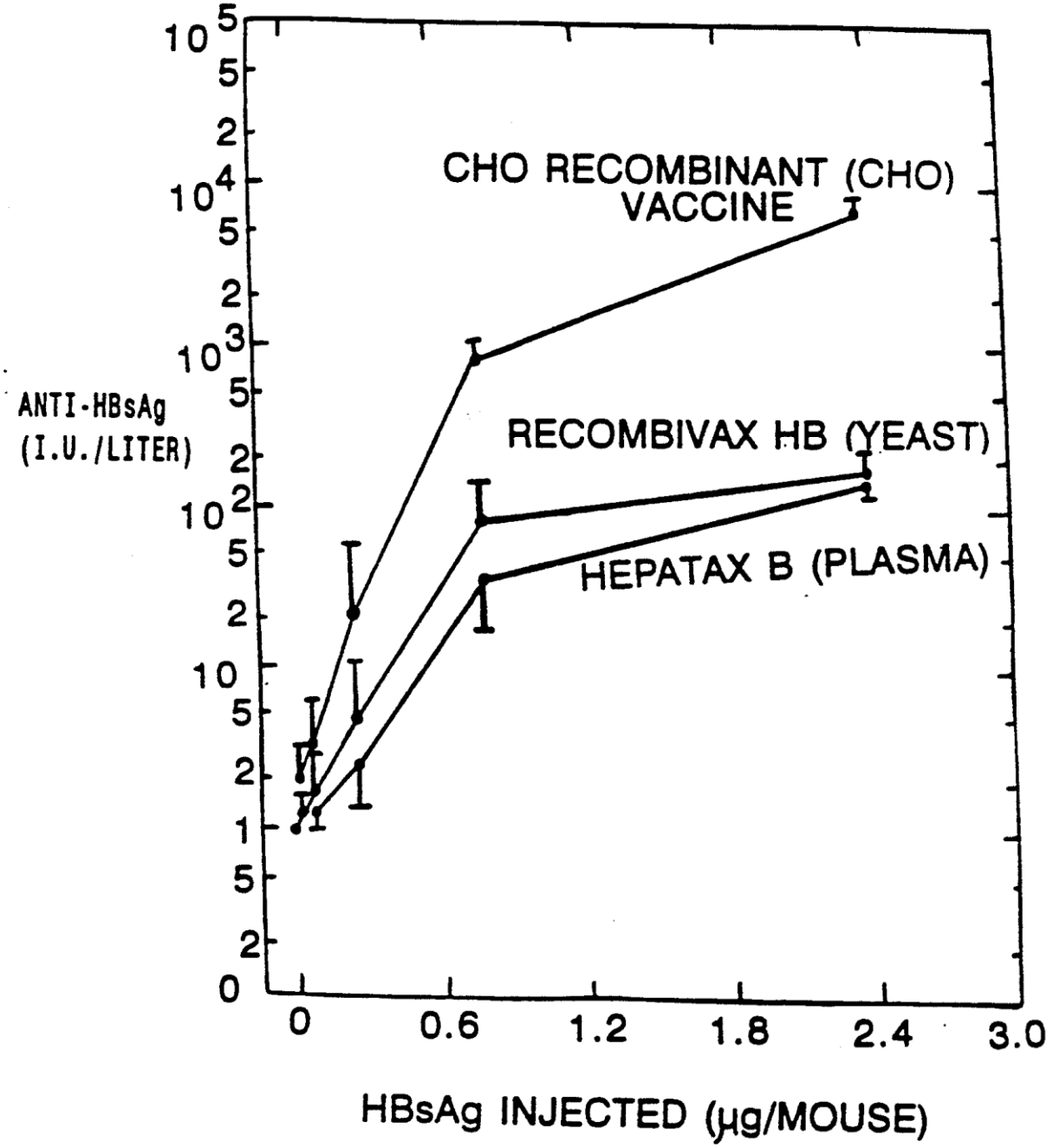

SUCROSE (%)
50
40
30
20
10
0
500
250
0
HBsAg (μg)
0
4
8
12
16
20
FRACTION No.

FIGURE 5

FIGURE 7


ANTI-HBsAg
(I.U./LITER)
CHO RECOMBINANT (CHO)
VACCINE
RECOMBIVAX HB (YEAST)
HEPATAX B (PLASMA)
HBsAg INJECTED (µg/MOUSE)
0
0.6
1.2
1.8
2.4
3.0

11.3 Overview: Required Devices for Engerix B Downstream Processing

I-centrifugation

II-grinding bead mill

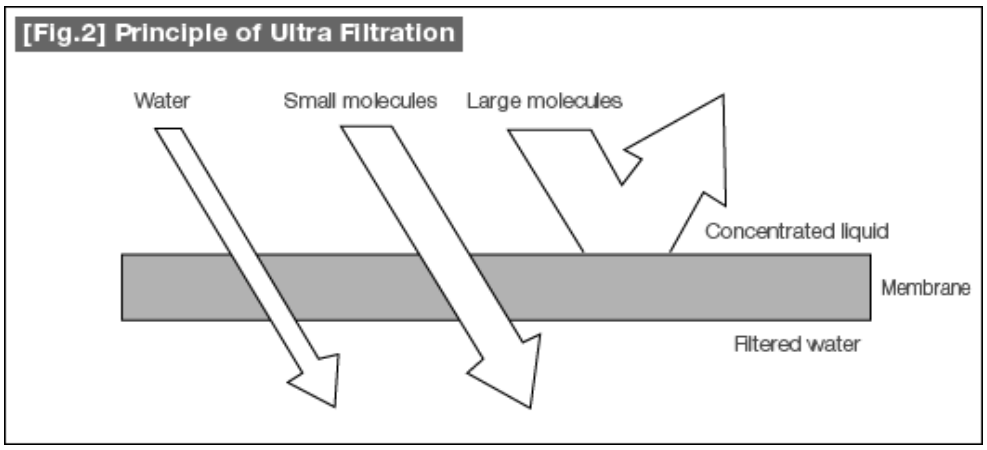
Link: http://www.omni-inc.com/omni-bead-ruptor-12-homogenizer-p-522.html

III- centrifugation

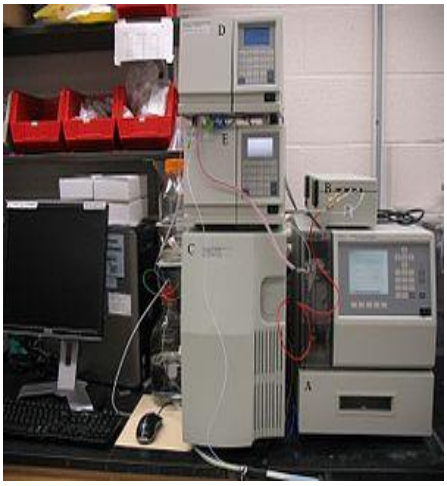
IV-centrifugation

V- ultrafiltration

link: http://www.solarisgroup.org/tytan-micro-ultra-filtration.html



VI- 4b gel permeation chromatography



chromatographic gel permeation column : A typical Waters GPC instrument including A. sample holder, B.Column C.Pump D. Refractive Index Detector E. UV-vis Detector

VII- anion exchange chromatography

chromatographic anion exchange column

VIII-ultracentifugation

link:http://www.medicalexpo.com/prod/beckman-coulter-international-sa/high-performance-bench-top-laboratory-centrifuges-75322-507590.html

IX-desalting gel permeation

desalting gel permeation

X-steril filtration

الالات اللازمة لعملية ال **Downstream** :

السعر	الشركة	المرحلة	الالة
...	...	I , III , IV	centifuge
...	...	V	ultrafiltration
...	...	VI	Chromatographic gel permeation columns
...	...	VII	Chromatographic anion exchange columns
...	...	VIII	ultracentrifuge
...	...	IX	Desalting gel permeation

11.4 Chromatographic Purification device – process scale

11.4.1 Purification system:

From "ÄKTAprocess™ Operating Instructions": Chapter 5 (Operation)

11.4.2 ÄKTAprocess

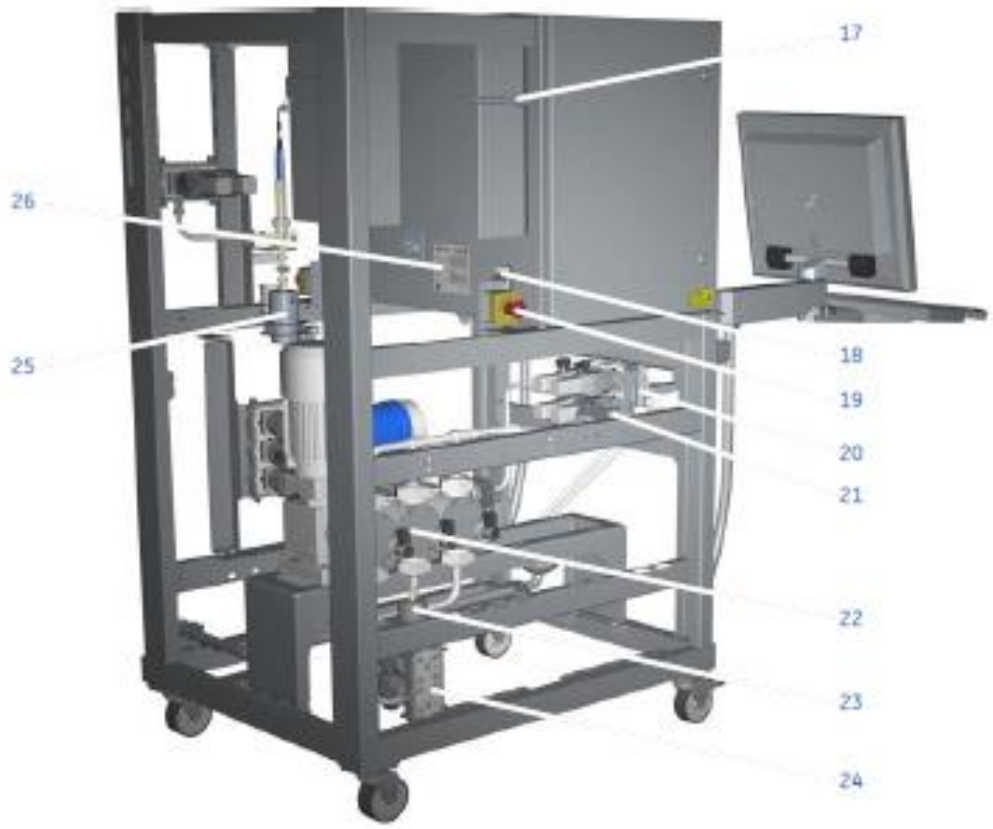
System with standard configuration: front view

The illustration below shows a front view of the standard configuration of ÄKTAprocess

Part	Function
1	pH probe
2	Battery limit: System outlets (2)
3	pH probe calibration holder
4	**EMERGENCY STOP**
5	Battery limit: Column 1 connections (2)
6	Flow meter
7	Battery limit: Common waste outlet
8	Swiveling wheel with brake (4)
9	Common waste collection cup
10	Pre-column pressure meter
11	Skid maneuvering handle (2)
12	Air trap
13	Operator console with keyboard and monitor
14	Indicator lamp - **ALARM**
15	Indicator lamp - **RUN/PAUSE**
16	Indicator lamp - **POWER**

rear view

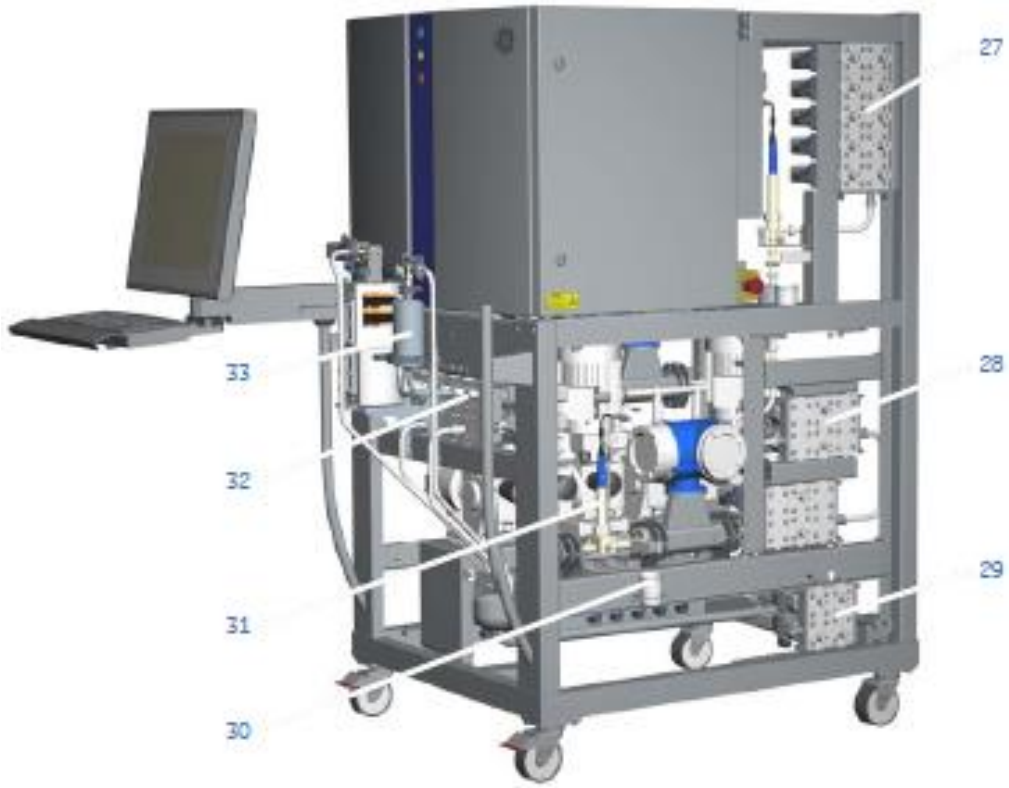
The illustration below shows a rear view of the standard configuration of ÄKTAprocess.



Part	Function
17	Pneumatic air supply connection port
18	SYSTEM POWER SWITCH
19	EMERGENCY STOP
20	Pressure meter
21	Pre-column conductivity meter
22	System pump A
23	Moveable air sensor
24	Battery limit: System inlets (2)
25	Post-column conductivity meter
26	System label

System with all options: front view

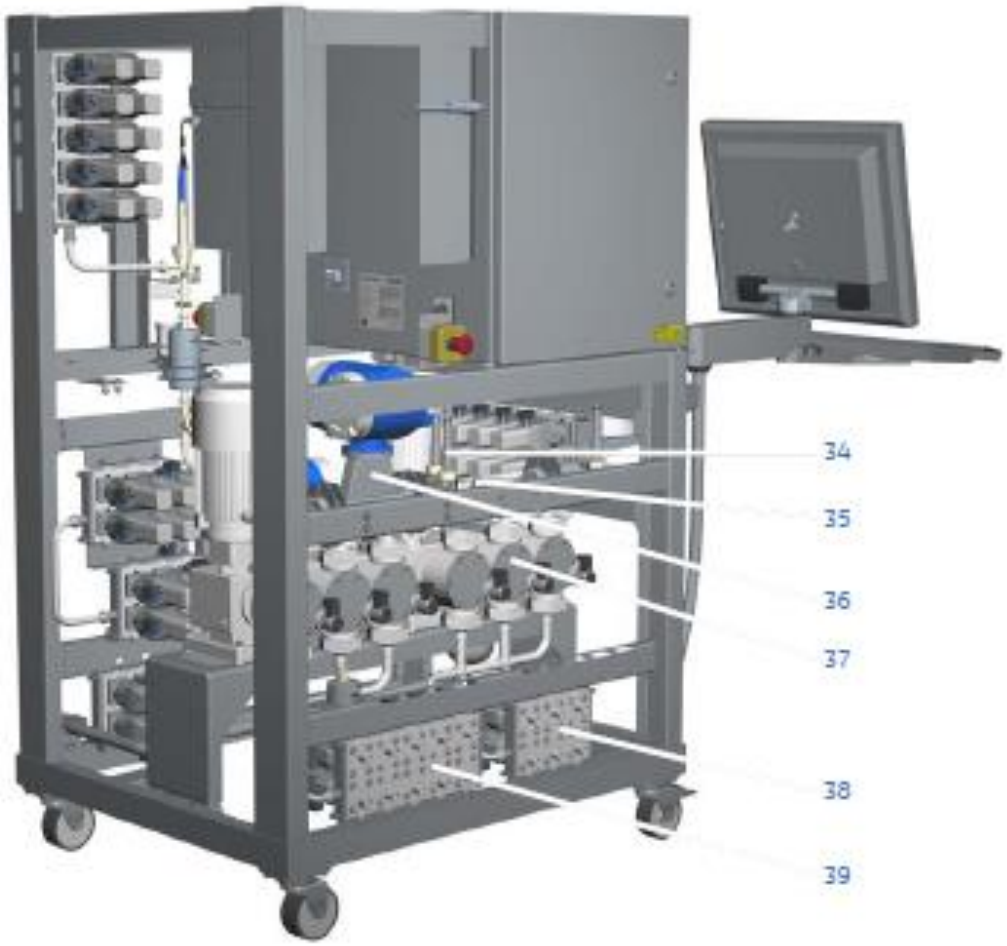
The illustration below shows a front view of an example of ÄKTAprocess with all optional Components



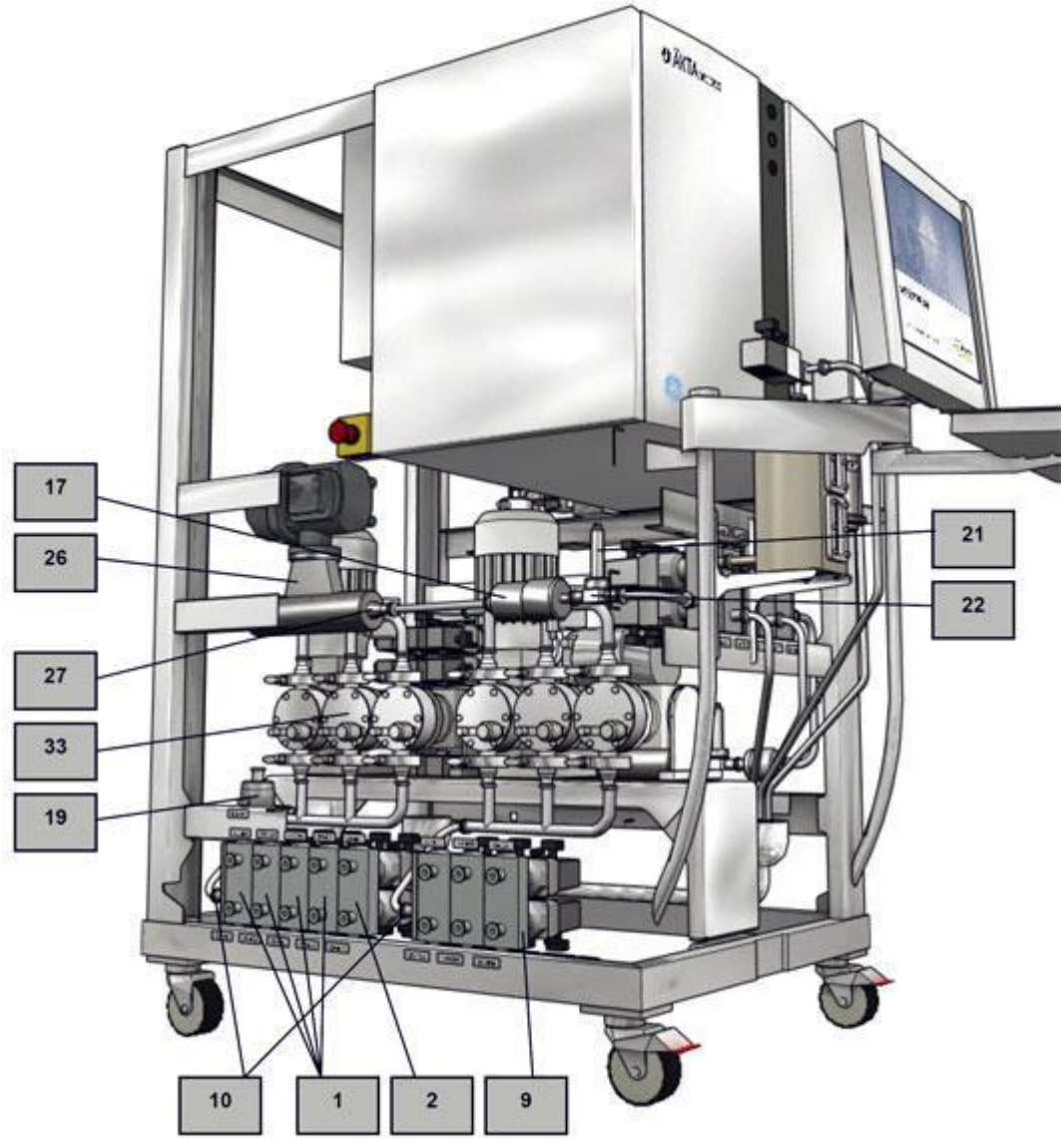
Part	Function
27	Battery limit: System outlets (10)
28	Battery limit: Column 2 connections (2)
29	Battery limit: CIP / AxiChrom™ valves
30	Pre-column pH probe calibration cup
31	Pre-column pH probe
32	Sample pump inlet
33	In-line filter

rear view

The illustration below shows a rear view of an example of ÄKTAprocess with all optional Components

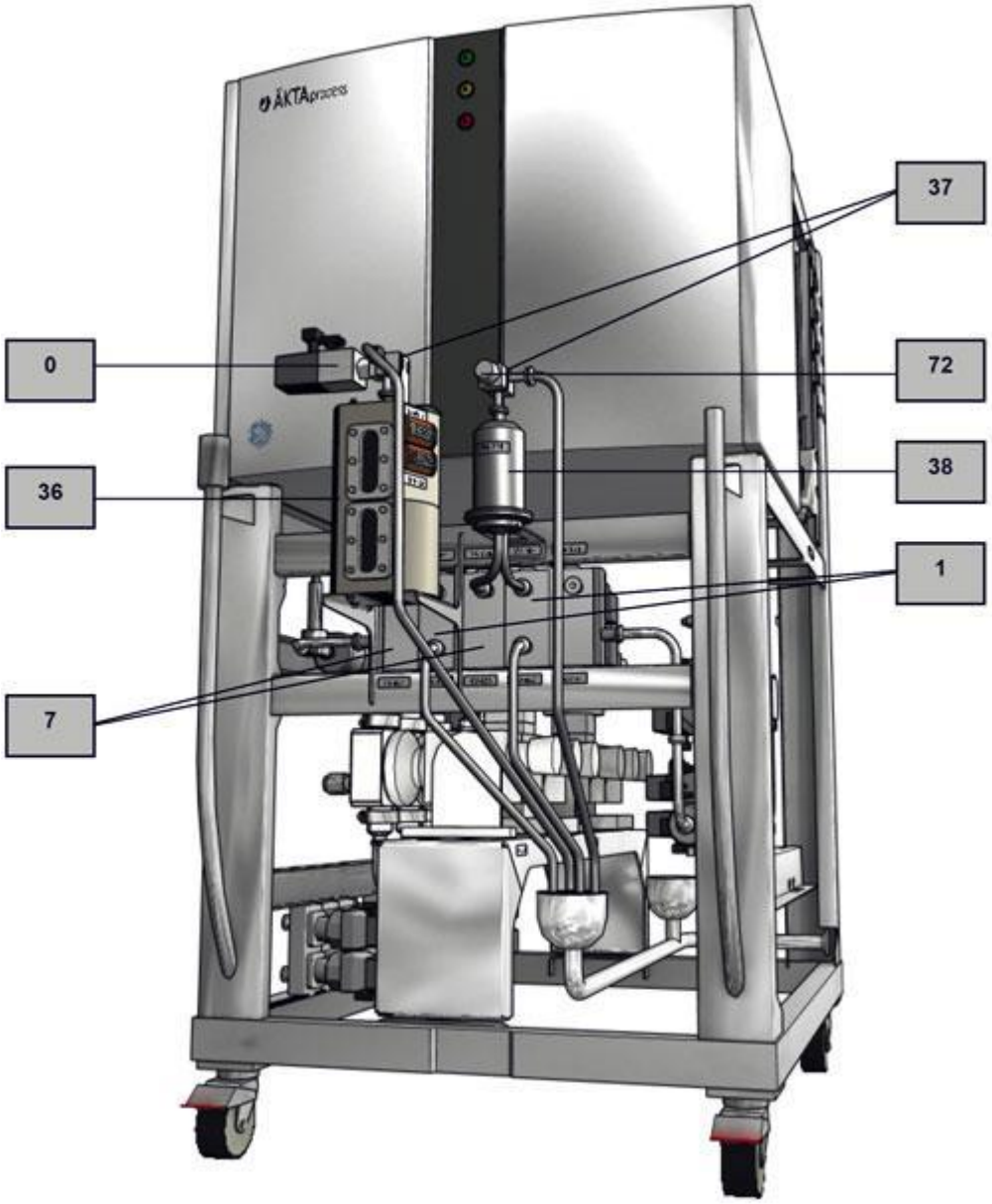


Part	Function
34	Pressure meter with PCV option (2)
35	Pressure control valve
36	Flow meter
37	System pump B
38	Battery limit: Buffer B inlets (6)
39	Battery limit: Buffer A inlets (10)



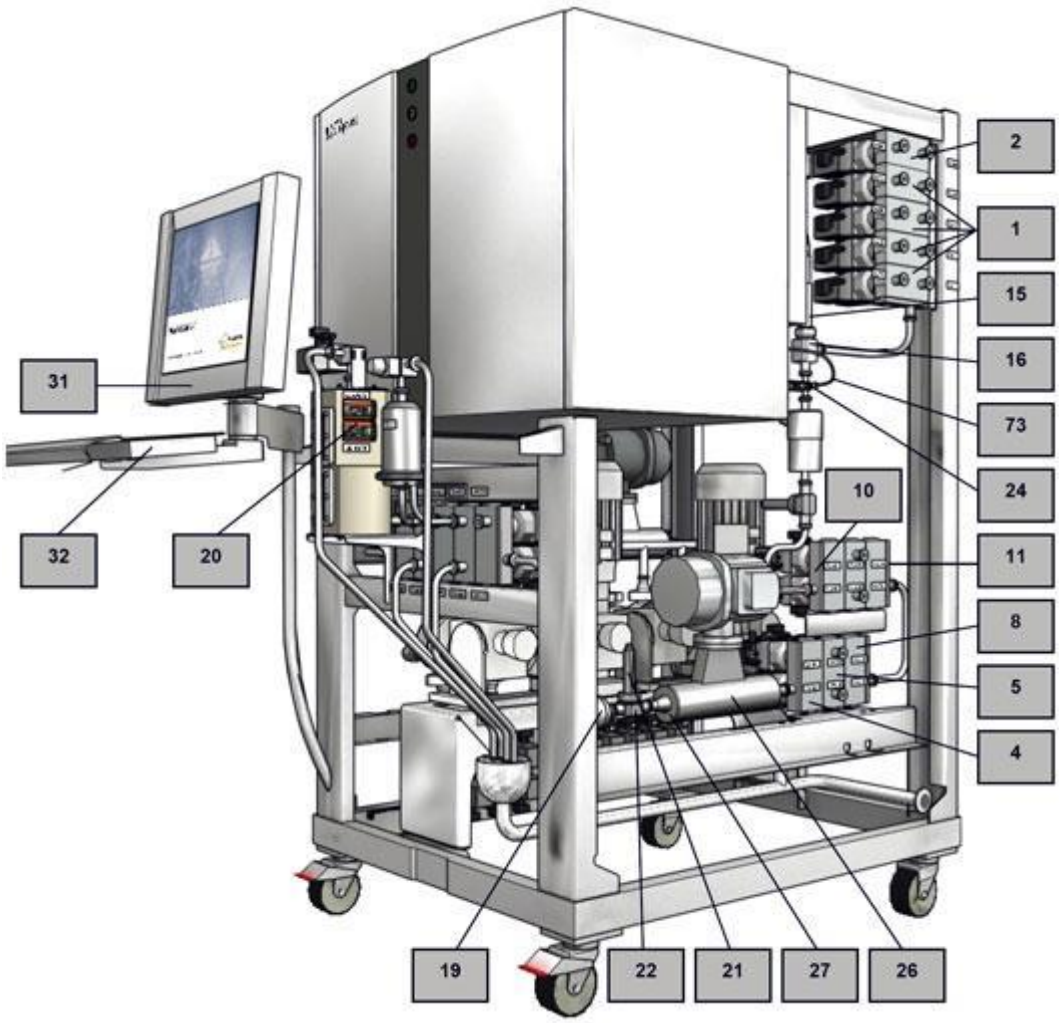
1	Valve body centre, i.d. 10 mm
2	Valve body right, i.d. 10 mm
9	Valve block mounting plate DN08, DN10
10	End connection left, i.d. 10 mm
12	Block rod 1, to valve bodies DN08 & DN10
12	Block rod 2, to valve bodies DN08 & DN10
12	Block rod 3, to valve bodies DN08 & DN10
12	Block rod 4, to valve bodies DN08 & DN10

12	Block rod 5, to valve bodies DN08 & DN10
17	Cable conductivity sensor Dsub-ODU, length = 2.2 m
17	Assembly / Disassembly tool to conductivity sensor
17	O-ring kit
17	Conductivity sensor,i.d. 8 mm
19	Cable air sensor RJ12-ODU6, length = 5.4 m
19	Cable air sensor RJ12-ODU6, length = 2.4 m
19	Air sensor i.d. 10 mm
21	Cable to pressure sensor RJ12-Binder
21	Cable to pressure sensor RJ12-Binder
21	Pressure sensor PAA35LXH 12bar
22	Pressure flow cell,i.d. 10 mm
22	O-ring 17.2x1.78 mm
26	Local display for flow meter
26	Flow meter DN10
27	TC adaptor, i.d. 10 mm
27	O-ring kit 17.04x3.53 mm, for TC adaptor
33	Clamp Tool CV Lewa Pumps
33	Pump head hygenic forpump 90 PP EPDM
33	Valvebody & guide kit90 PP
33	Wear & Tear Kit
33	Pump Hygienic 3x90 PP EPDM



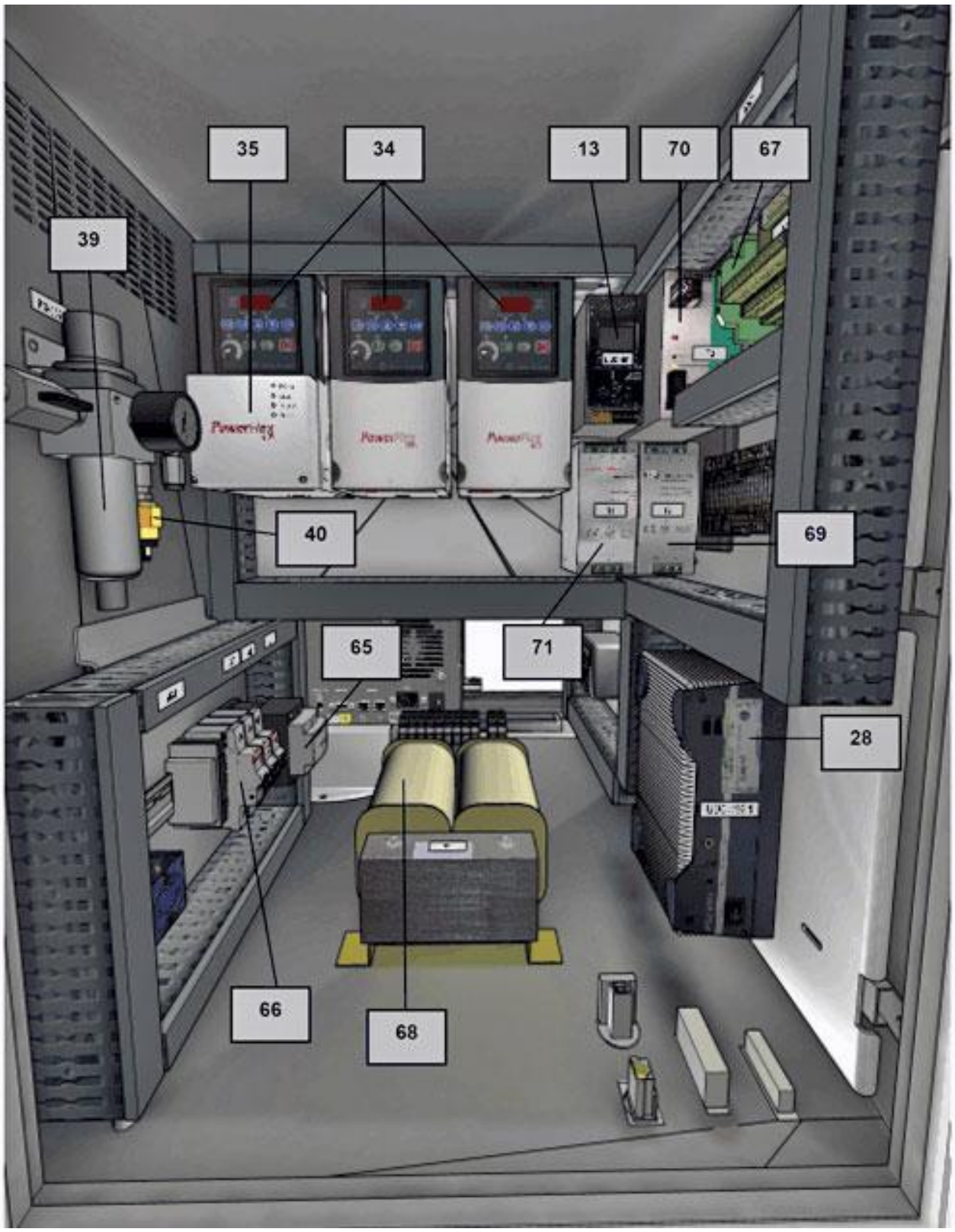
1	Valve body centre, i.d. 10 mm
7	Actuator DN10 90
7	Valve body air trap, i.d. 10 mm
12	Block rod 1, to valve bodies DN08 & DN10
12	Block rod 2, to valve bodies DN08 & DN10
12	Block rod 3, to valve bodies DN08 & DN10
12	Block rod 4, to valve bodies DN08 & DN10
12	Block rod 5, to valve bodies DN08 & DN10
36	O-ring kit for air trap 10 mm
36	Sight glass 34x95x17 mm
36	Air trap, i.d. 10 mm

37	Actuator DN08 0
37	Valve body angled right, i.d. 3 mm
37	Diaphragm DN08
38	Clamp, 3 inch TC
38	Gasket, 91 mm TC, i.d. 73 mm
38	Filter house, i.d. 10 mm
38	Filter Cartridge
72	Actuator manual valve DN08



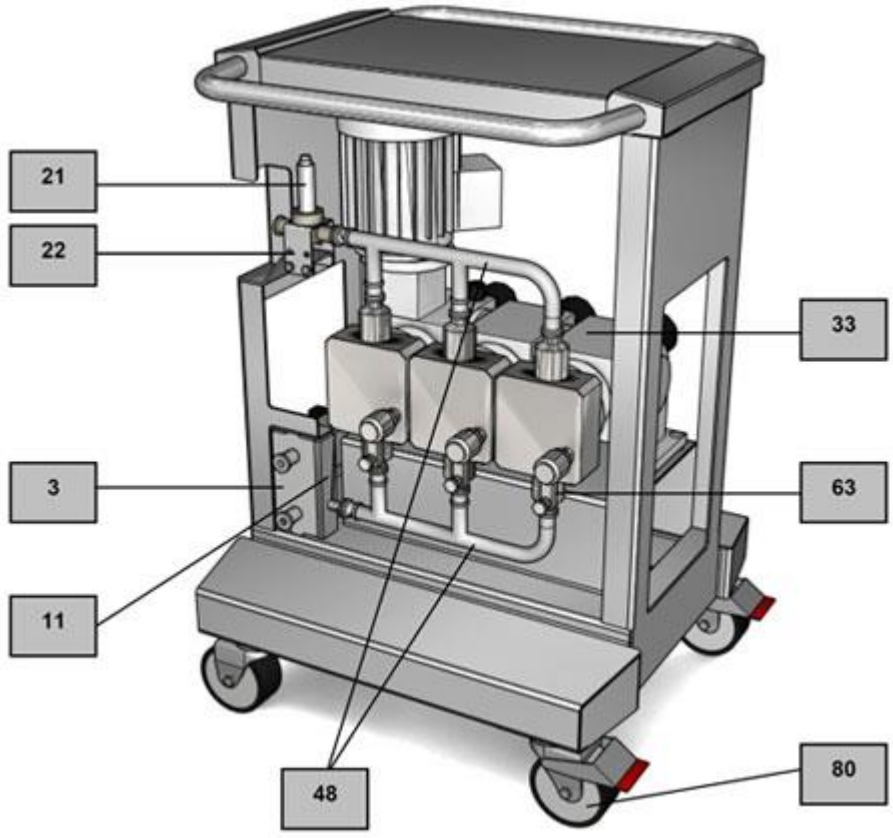
1	Valve body centre, i.d. 10 mm
2	Valve body right, i.d. 10 mm
4	Valve body right, i.d. 10 mm
5	Valve body centre, i.d. 10 mm
8	Valve body left, i.d. 10 mm

10	End connection left, i.d. 10 mm
11	End connection right, i.d. 10 mm
12	Block rod 1, to valve bodies DN08 & DN10
12	Block rod 2, to valve bodies DN08 & DN10
12	Block rod 3, to valve bodies DN08 & DN10
12	Block rod 4, to valve bodies DN08 & DN10
12	Block rod 5, to valve bodies DN08 & DN10
15	pH cable CPK9 TOP 68, length = 5 m
15	pH electrode
16	Adaptor pH flow cell
16	Adaptor nut
16	Cleaning-in-place (CIP) Cap to pH flow cell
16	pH flow cell
19	Cable air sensor RJ12-ODU6, length = 5.4 m
19	Cable air sensor RJ12-ODU6, length = 2.4 m
19	Air sensor i.d. 10 mm
20	Level sensor capasitive IFM
21	Cable to pressure sensor RJ12-Binder
21	Cable to pressure sensor RJ12-Binder
21	Pressure sensor PAA35LXH 12bar
22	Pressure flow cell,i.d. 10 mm
22	pH and pressure flowcell i.d. 10 mm
22	O-ring 17.2x1.78 mm
24	UV flow cell, i.d. 1 inch
24	UV flow cell, i.d. 8 mm
24	O-ring kit
26	Local display for flow meter
26	Flow meter DN10
27	TC adaptor, i.d. 10 mm
27	O-ring kit 17.04x3.53 mm, for TC adaptor
31	Console complete without touch screen
31	Console complete with touch screen
32	Keyboard UK/US IP65
73	Long optical fibre kit, 500 mm



13	Profibus/AS-I gateway
13	Wire ASI-cable 2-port buscable, length = 13 m
13	Connector ASI-cable
28	Computer AAEON AEC-6850

28	Communications cable RJ45-RJ45/IP67, length = 10 m
34	Frequency converter 0.75kW / 1ph
35	Frequecy converter profibus interface
39	Pressure regulator
40	Pressure switch PM11-NA
65	Contactor 25A 24V DC
66	Fuse 10 A, 1 POL
67	Opto Coupler DEK-OE-24DC
68	Trafo Mains 2000VA 1-PHASE
69	Power supply AC/DC 24V 75W DIN
70	Power supply ASi, 30 VDC 2.4 A
71	Power supply AD/DC 12V 75 W DIN



3	Actuator DN10 0
3	O-ring, DN10
3	Block rod 1, to valve bodies DN08 & DN10

3	Pneumatic quick fitting T M5 / i.d. 6 mm
3	Pneu. quick fitting (L)M5 / i.d. 6 mm
3	Diaphragm DN10
3	Valve body left, i.d. 10 mm
11	End connection right, i.d. 10 mm
21	Cable to pressure sensor RJ12-Binder
21	Cable to pressure sensor RJ12-Binder
21	Pressure sensor PAA35LXH 12bar
22	Pressure flow cell,i.d. 10 mm
22	O-ring 17.2x1.78 mm
33	Clamp Tool CV Lewa Pumps
33	Pump head hygenic forpump 90 PP EPDM
33	Valvebody & guide kit90 PP
33	Wear & Tear Kit
33	Pump Hygienic 3x90 PP EPDM
48	Pump manifold, i.d. 10 mm
63	TC clamp kit, 25 mm TC
63	O-ring, DN10
63	TC gasket 25 mm, i.d. 15 mm
63	TC Gasket, 25 mm, i.d. 16.5 mm
63	End cap, 25 mm TC
63	TC-gasket kit, i.d. 10 mm
80	Wheel kit

3.1.1.12. Structural components

Electric cabinet

The electric cabinet serves as the container for all electrical and pneumatic equipment.

Skid

The rigid stainless steel structure supports all process components and the electric cabinet.
The structure is designed for handling in a production environment and to be easy to move and keep clean.
The steel structure protects all installed components while still allowing easy access.
The structure occupies a small box-shaped space that makes it easy to fit into any location in the production facility.

Control system

ÄKTAprocess is fully automated by means of the UNICORN control system. Once the required

methods are created and approved, a non-expert user can safely operate the system.

Control unit

A **CU-960** control unit is the controlling interface between UNICORN and the components of ÄKTAprocess.

The **CU-960** control unit is located inside the cabinet.

Computer

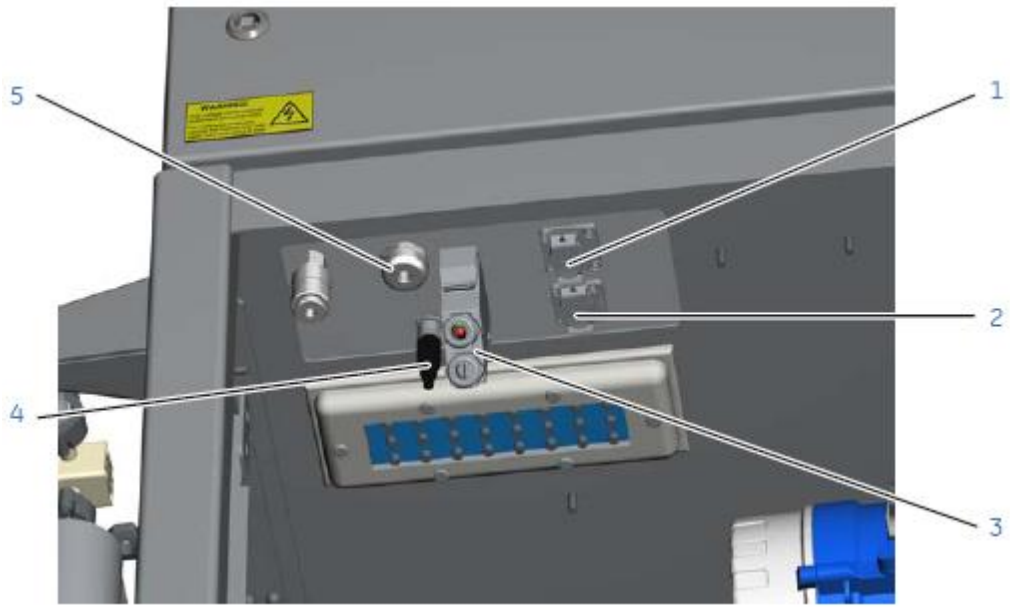
The computer is built into the cabinet and fully protected from the outside environment.

User console

The display and input equipment is ergonomically designed for usage in a clean production environment.

Communications

Communication with most controlled components mounted outside the cabinet uses the PROFIBUS™ industry standard communication protocol and hardware. The PROFIBUS connection and other communication ports are located on the underside of the electrical cabinet, as shown in the illustration below. For information on where to connect the PROFIBUS signal cable to the AxiChrom Master, see the *AxiChrom User Manual*



Part	Function
1	USB connection port
2	Ethernet connection port
3	Customer I/O connection
4	PROFIBUS connection
5	UPS Power

Inlets and outlets

This section describes the inlets and outlets, including the drain outlet, of ÄKTAprocess that are provided in the standard configuration.

Connections

The standard configuration of ÄKTAprocess has two inlets, two outlets and connections for one column. As shown in the flowchart in *Section 3.5 Flowchart, on page 72* ÄKTAprocess has a moveable air sensor that may be connected to any inlet.

For standard ÄKTAprocess configurations the pressure on the inlets should be in the range 0 to 0.2 bar. The outlets can handle backpressure up to 1 bar.

Drains

All drains from ÄKTAprocess are collected to a single drain outlet. The drains are first collected in an open cup to ensure that no back pressure is applied on any parts of the processing system.

Meters and sensors

This section describes the meters and sensors that are installed as standard components of ÄKTAprocess.

Overview

ÄKTAprocess is provided with a set of sensors and meters that provide data to the control system, enabling it to control the progress and detect the performance of the process in a satisfactory way.

The basic system setup includes meters and sensors that measure pressure, flow, conductivity (Cond), pH, air, temperature and UV. Measurement of these parameters enables basic isocratic operation and the air sensor before the column also makes sure that no air enters the column during processing.

Flow meter measurement principle

The measuring principle of the flow meter is based on the controlled generation of Coriolis forces. Refer to the flow meter manual in the product documentation package for more information.

System pump

This section describes the basic ÄKTAprocess system pump. In the standard configuration, a single system pump is provided that supports isocratic operation.

Pump type

The system pump is a triple head diaphragm pump, or a 5-headed diaphragm pump for flow rates 45 to 2000 l/h. The process wetted parts of the pump heads are effectively sealed from non-sanitary components of the pump.
The pump is provided with stroke length adjustment knobs. These are factory preset at delivery and must not be adjusted by the user.

Pump stroke frequency

The pump stroke frequency is controlled by the flow that is set in the UNICORN control software.

Safety monitoring

The system is protected from exceeding the high pressure limit by the electronic module **ALP-900**, an air, level and pressure monitoring system that is situated inside the cabinet.
The **ALP-900** monitors:
1 The pressure in each pressure sensor
2 The pressure difference between each pressure sensor
3 The temperature in the process liquid
If any of the monitored parameters reaches a critical limit, the **ALP-900** will shut down the pumps independently from the UNICORN control system.

Air trap

An air trap is installed in the flow path of ÄKTAprocess. This section describes the air trap and the sensors that are used for liquid level control.

Air trap function

The function of the air trap is to de-gas buffers. A vortex is created in the air trap and the liquid in the air trap is pressed downwards and outwards by the centrifugal force generated while air is separated in the center of the chamber. The rotation eliminates pockets of stagnant liquid, which prevents unwanted build-up of solids (e.g., bacterial cells) and simplifies the cleaning of the air trap.

Level sensors

Two sensors for automatic liquid level control are installed in the air trap. This sensor assembly consists of a high and a low level sensor. The sensors must be re-calibrated if the LED indicator displays a red light while the liquid level is still far from the low or high level markers.
It is recommended that after a power down a calibration of the level sensors should always be performed. See Section 6.6.2 Air trap calibration, on page 153.
Note:

When the air trap is filled with liquid that foams easily, for example liquids containing detergents and protein solutions (sample), large volumes of air should not be allowed to enter into the air trap. If foam is formed it may interfere with the automatic liquid level control.

Note:

Always position a movable air sensor at the inlet of the sample or detergentcontaining liquid. The air sensor will set the system to **Pause** when air is detected, which will prevent the build up of foam in the air trap or can trigger the next step in the method.

3.1.1.13.Valves

General description

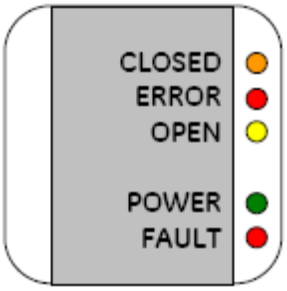
With the exception of the filter housing options that have either one or three manual air outlet valves, all valves are diaphragm valves that are actuated by compressed air. The valve actuators are controlled by an ASi bus.

The inlet and outlet valve configurations are identical. Each valve consists of a valve body, a diaphragm and an actuator. Two valves are combined into a valve block.

Due to their size and weight, the valves for 1" systems are mounted in turnable cradles.

Valve LED indicator lights

The valve LED indicator lights are illustrated below.



Label	Color	Description (when applicable)
CLOSED	Orange	Steady light: Actuator in closed position
ERROR	Red	Steady light: Programming, sensor or internal error
OPEN	Yellow	Steady light: Actuator in open position
POWER	Green	Voltage on
FAULT	Red	Steady light: Slave address error

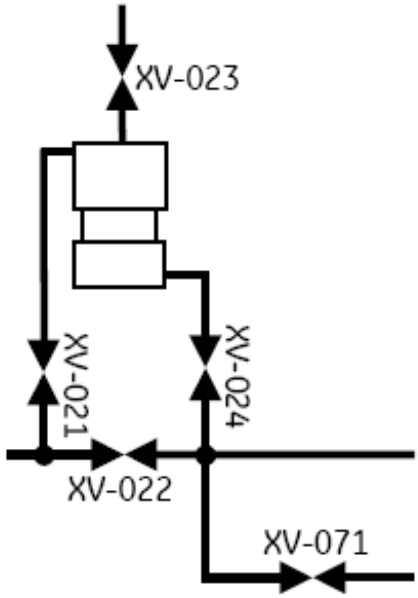
Valve default positions

When the system is powered up and connected to compressed air, the default positions for the various valves are given in the table below. If no control signal is present, for example if the mains power is shut off, the valves will revert to ***Closed*** positions.

Air trap valves

The air trap valve blocks are directly connected, either to the optional filter valve blocks or to tubing going to the pre-column air sensor, in order to minimize the dead volume

that is caused by connecting valve blocks with tubing.
The layout of the air trap valves is illustrated below.



Valve positions	Open valve(s)
Bypass	XV-022
Inline (Default)	XV-021 + XV-024
Fill	XV-021 + XV-023
Fill_Inline	XV-021 + XV-023 + XV-024
Out_through_drain	XV-021 + XV-024 + XV-071
Drain (No flow)	XV-023 + XV-024 + XV-071

The ***Drain*** valve position is used for example when the air trap is emptied before disassembly or to lower the liquid level in the air trap. The pump(s) must be set to 0.0 l/h when the ***Drain*** valve position is used.

Instruction	Setting
Valves:AirTrap	*Bypass*
	Fill
	Fill_Inline
	Drain
	Out_through_drain

The instruction ***System:Settings:Specials:AirTrapPauseFunction*** defines if the valve goes back to the default position (Inline) or if it remains in position when the system is

set to Pause.

Sample connection valves

The optional sample pump is connected to the sample connection valve, where the feed from System pump A and the optional gradient pump B is also connected, after the air trap and the optional filter. Sample inlet valves are available only on systems that are delivered with a sample pump.
When an inlet valve is open (A, B or Sample inlet valves), the corresponding sample connection valve will also open. The sample connection valves cannot be controlled independently by the operator.

Note:
The alarm for the sample inlet valves must be disabled if the sample pump is disconnected. See Section5.1.5 Final checks, on page 123 for UNICORN settings.

Column valves

The column valve sets (column 1 and the optional column 2) each consist of six valves. Similar to the inlet valve blocks, the blocks are connected directly to each other to enable the shortest possible flow path.

3.1.1.14.Optional components

Extra system pump inlets

Up to eight extra inlets with individually controlled valves can be installed.
This means that ÄKTAprocess is able to manage up to ten individual inlets for system pump A.

Extra system outlets

Up to eight extra system outlets with individually controlled valves can be installed.
This means that ÄKTAprocess is able to manage up to ten individual outlets in total.

System pump B

ÄKTAprocess can be provided with a second system pump. The addition of a second system pump enables ÄKTAprocess to operate as a gradient system.
The B-pump can be provided with up to six individually controlled inlets.
If the System pump B option is selected, an extra flow meter can also be provided to enable the individual pump flows, as well as the total system flow, to be measured.
The B-pump type is identical to the A-pump.

In-line filter

A filter can be installed between the air trap and the column to prevent foreign objects from contaminating the column. Different types of in-line filter are available, including a disposable capsule filter option. Filter housings may be made of steel or polypropylene.

Two columns

ÄKTAprocess can be configured to incorporate a second column. With this option, the ÄKTAprocess can supply two columns, one after the other with mobile phase.

Sample pump

The sample pump allows sample to be injected into the column without the need to use any of the system pumps for this purpose.
There are two optional inlets feeding the sample pump. The sample pump is also provided with an extra pressure meter that protects the system against over pressure.

Extra pressure meter

An extra pressure meter can be installed after the column to accurately measure the pressure drop over the column.

Extra pH meter

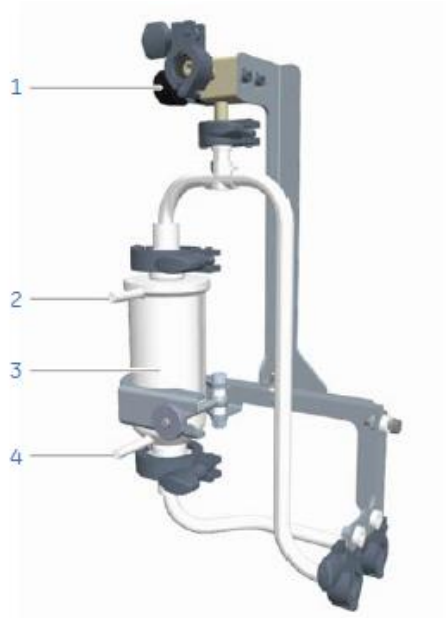
An extra pH-meter can be installed before the column on a gradient system to enable the gradient to be monitored.

Filter valves

The optional in-line filter valve block set is identical to the air trap valve block set. There is also a manual valve for air evacuation, HV-301.
The layout of the filter valves is illustrated in the following diagram.

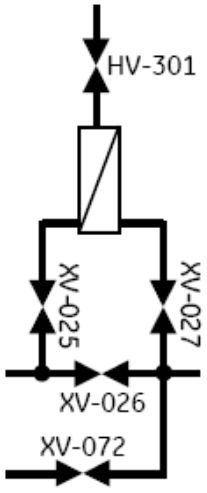
Capsule filter and valves

A disposable capsule filter option may be selected instead of the in-line filter option described above. The disposable capsule filter housing is made of polypropylene and includes two additional manual valves for air evacuation, as illustrated below.



Part	Function
1	Manual valve **HV-301**
2	Outlet to manual valve **HV-303**
3	Capsule filter housing
4	Outlet to manual valve **HV-302**

The layout of the capsule filter valves is illustrated in the following diagram.



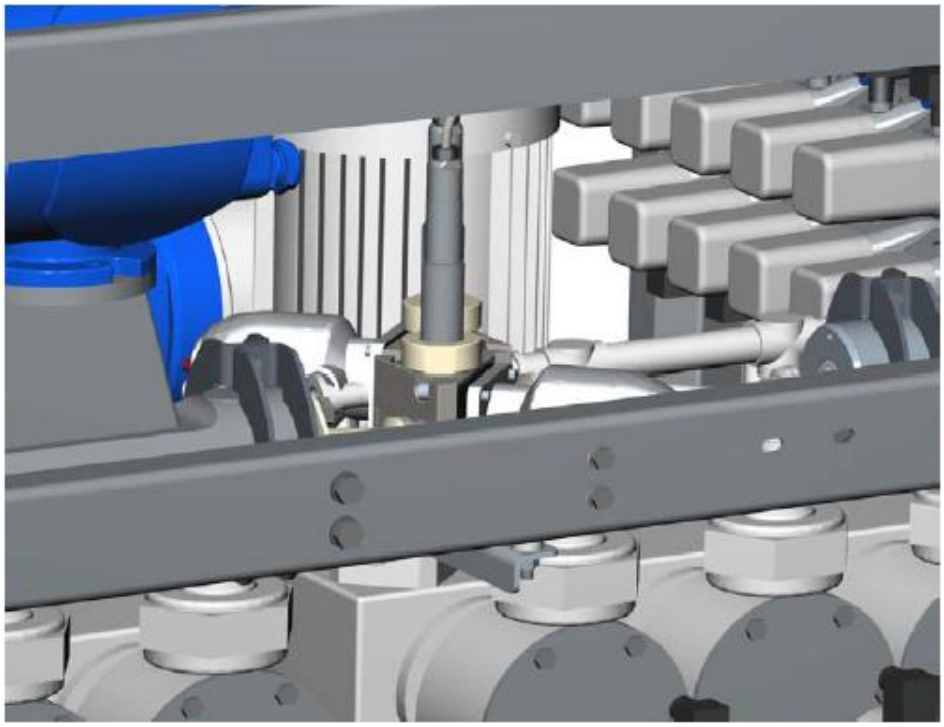
Valve positions	Open valve(s)
Bypass (default)	XV-026
Inline	XV-025 + XV-027
Out_through_drain	XV-025 + XV-027 + XV-072
Drain (No flow)	HV-301 + XV-027 + XV-072

The ***Drain*** valve position is used, for example, when the filter housing is emptied before

replacing the filter. The pump(s) must be set to 0.0 l/h when the ***Drain*** valve position is used.

Pressure control valves (PCV)

ÄKTAprocess can be provided with up to two optional pressure control valves, **PCV-341** and **PCV-342**, as shown in the illustration below.



The function of the PCVs is to protect the system from 'free flow' if the inlets are fed with a higher pressure than 0.2 bar.
The pressure control valve option allows the pressure on the inlets to be regulated and the flow through the system via individual system pumps to be controlled.

ALP2 PCV safety monitoring

If ÄKTAprocess is optionally configured to include a pressure control valve, or valves, the system will also be equipped with an **ALP2** air, level and pressure monitoring system.
The **ALP2** unit protects against exceeding maximum operating pressures by monitoring pressure between the pumps and the PCVs.

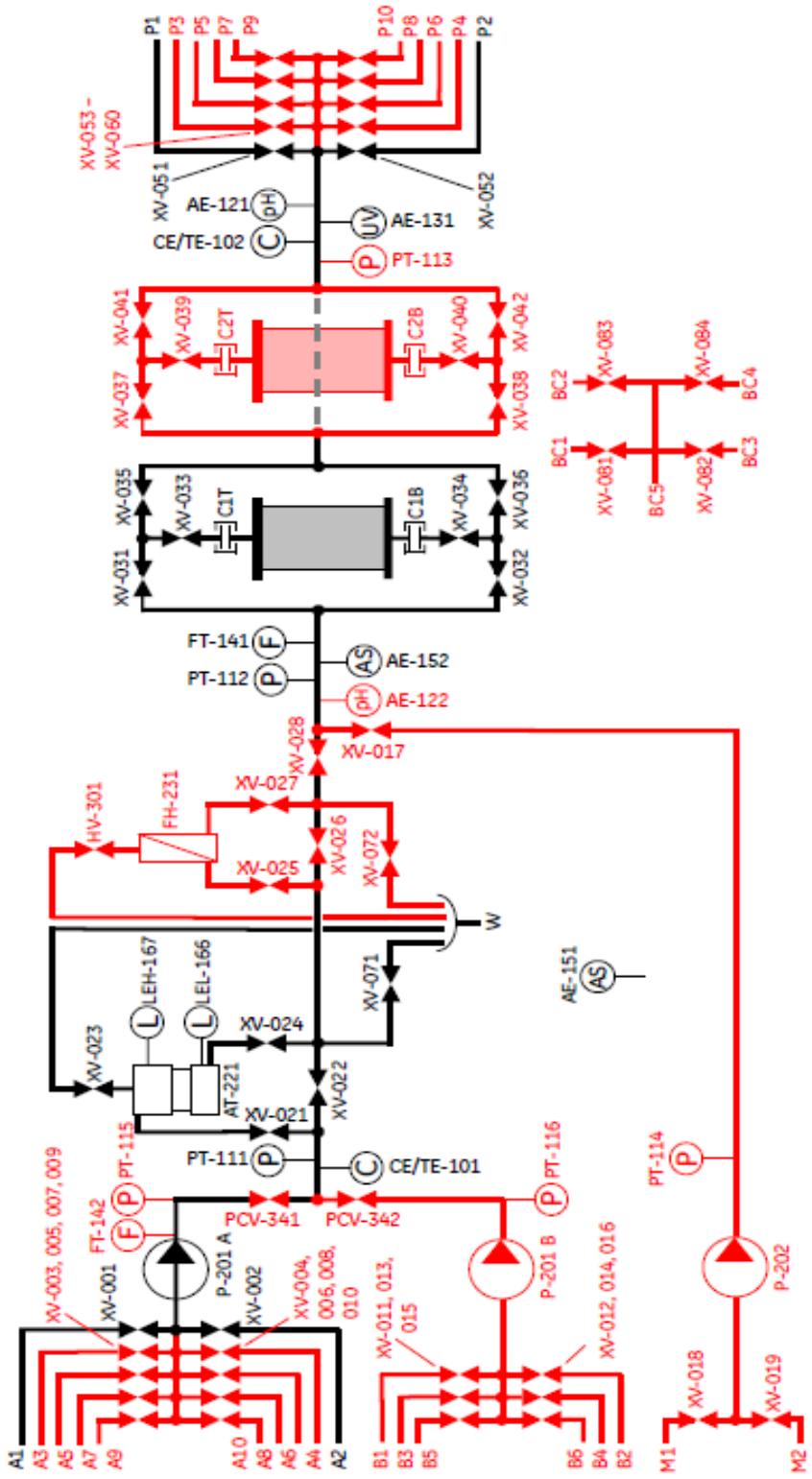
CIP / AxiChrom manifold

A CIP / AxiChrom manifold with four individually controlled valves enables UNICORN to control CIP with up to four inlets and control processing together with a connected AxiChrom column.
See *Using the CIP/AxiChrom manifold option, on page 139* for more information about connections for CIP. For Intelligent Packing with AxiChrom columns, see AxiChrom manuals for details on connection.

Anybus™ X-gateway

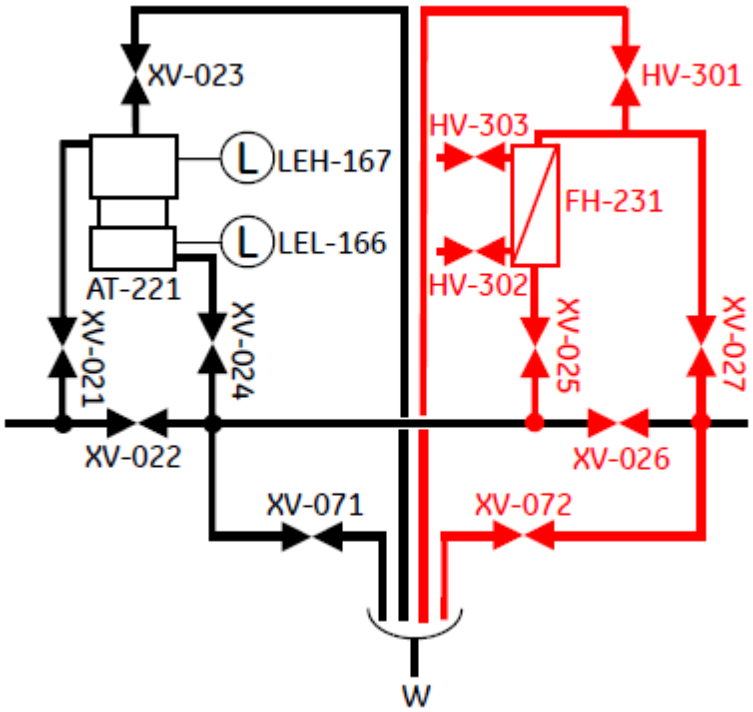
The system can be provided with an optional Anybus X-gateway to enable communication between ÄKTAprocess and the customer network. The signals transferred can both be analog process readings, digital status and handshake signals. The Anybus X-gateway is located inside the electrical cabinet. The interface to the gateway is PROFIBUS Slave through a M12 connector in the bottom of the electrical cabinet.
The Anybus X-gateway copies I/O-data in both directions, thus enabling data exchange between two optically isolated PROFIBUS networks. The Anybus X-gateway connections can be used for many different applications. See the PROFIBUS Communication Interface documenation for a description of how the different I/O-data is addressed in the memory space.

3.1.1.15.Flowchart



Capsule filter option

The illustration below shows the corresponding air trap and filter block alternative section of the flowchart if the capsule filter option is selected.



Note: *Black represents standard components; Red represents optional components*

3.1.1.16.Process components

The following table lists the process components that are shown in the flow chart.

Tag	Function (qty)	Note
1, 2	Outlets	
3 to 10	Outlets	Optional
A1, A2	Buffer A inlets	
A3 to A10	Buffer A inlets	Optional
AT-221	Air trap	
B1 to B6	Buffer B inlets	Part of system pump B option
C1T	Column 1 top connection	

Tag	Function (qty)	Note
C1B	Column 1 bottom connection	
C2T	Column 2 top connection	Part of column 2 option
C2B	Column 2 bottom connection	Part of column 2 option
CIP1 to CIP4	CIP inlets	
CIP C	CIP common inlet	
FH-231	Filter	Option
HV-301	Filter vent valve	Part of filter option, manual
HV-302	Capsule filter bottom manual valve	Capsule filter option only
HV-303	Capsule filter top manual valve	Capsule filter option only
M1, M2	Sample inlets	Part of sample pump option
P-201 A	System pump A	
P-201 B	System pump B	Option
P-202	Sample pump	Option
PCV-341	Pressure control valve, A inlets	Option
PCV-342	Pressure control valve, B inlets	Option
W	Common waste	
XV-001, XV-002	Buffer A inlet valves	
XV-003 to XV-010	Buffer A inlet valves	Optional
XV-011 to XV-016	Buffer B inlet valves	Part of system pump B option
XV-017	Sample connection valve	Part of sample pump option
XV-018, XV-019	Sample inlets valves	Part of sample pump option
XV-021	Air trap inlet valve	
XV-022	Air trap bypass valve	
XV-023	Air trap vent valve	
XV-024	Air trap outlet valve	
XV-025	Filter inlet valve	Part of filter option

Tag	Function (qty)	Note
XV-026	Filter bypass valve	Part of filter option
XV-027	Filter outlet valve	Part of filter option
XV-028	System connection valve	Part of sample pump option
XV-031	Column 1 top inlet valve	
XV-032	Column 1 bottom inlet valve	
XV-033	Column 1 top valve	
XV-034	Column 1 bottom valve	
XV-035	Column 1 top outlet valve	
XV-036	Column 1 bottom outlet valve	
XV-037	Column 2 top inlet valve	Part of column 2 option
XV-038	Column 2 bottom inlet valve	Part of column 2 option
XV-039	Column 2 top valve	Part of column 2 option
XV-040	Column 2 bottom valve	Part of column 2 option
XV-041	Column 2 top outlet valve	Part of column 2 option
XV-042	Column 2 bottom outlet valve	Part of column 2 option
XV-051, XV-052	Outlet valves	
XV-053 to XV-060	Outlet valves	Optional
XV-071	Air trap drain valve	
XV-072	Filter drain valve	Part of filter option
XV-081 to XV-084	CIP / AxiChrom manifold	Option

3.1.1.17.Meters and sensors

The following table lists the meters and sensors that are shown in the flow chart.

Tag	Function	Note
AE-151	Buffer inlet air sensor	Movable
AE-152	Pre-column air sensor	Final check that no air enters the column
AT-121	Post-column pH-meter	
AT-131	Post-column UV-meter	Peak detection
CE/TE-101	Pre-column conductivity meter	Also includes a temperature meter
CE/TE-102	Post-column conductivity meter	Peak detection and CIP-control, also includes a temperature meter
FT-141	System flow meter	Measures the total system flow
LEH-167	Air trap high level meter	
LEL-166	Air trap low level meter	
PT-111	Pre-filter pressure meter	Option
PT-112	Pre-column pressure meter	Guards the column from over pressure, detects clogged column
PT-114	Sample pump pressure meter	Part of sample pump option
PT-115	PCV pressure meter, A inlets	part of PCV option
PT-116	PCV pressure meter, B inlets	part of PCV option

3.1.1.18. Connect a column

Considerations for AxiChrom columns

AxiChrom columns can be packed using ÄKTAprocess and Intelligent Packing.

Small diameter columns, up to 200 mm diameter, use ÄKTAprocess pumps to drive the adapter hydraulically.

Larger AxiChrom columns, 300 mm diameter and above, use an AxiChrom Master connected to the ÄKTAprocess Profibus connector.

See respective AxiChrom column manuals for information on hose connections.

For more details regarding using AxiChrom with ÄKTAprocess, refer to the AxiChrom user manual.

Preparations

The air sensor alarm(s) before the column must be disabled before filling the system/column with liquid (***Alarms:Air_Alarm:Disabled***).

Connect an empty column

Follow the instruction below to connect an empty column to ÄKTAprocess.

Step Action

1 Connect tubing between the system valve marked **COLUMN BOTTOM** and the column bottom.

2 Connect tubing between the system valve marked **COLUMN TOP** and the column top.

Connect a packed column without bypass lines/valves

Follow the instruction below to connect a pre-packed column without by-pass lines/valves to ÄKTAprocess.

Step	Action
1	Connect tubing to the system valve marked **COLUMN BOTTOM**, but do not connect the other end of the tubing to the bottom of the column at this time.
2	Set the system column valves to the ***UpFlow*** position.
3	Using the pump, fill the system with an appropriate liquid for column installation.
4	When the system, including the tubing connected to the column bottom system valve, is filled with liquid, connect the other end of the tubing to the bottom of the column.
5	Connect tubing between the top of the column and the system valve marked **COLUMN TOP.**

Connect a packed column with bypass lines/valves

Follow the instruction below to connect a pre-packed column with bypass lines/valves to ÄKTAprocess.

Step	Action
1	Connect tubing between the column bottom and the system valve marked **COLUMN BOTTOM**.
2	Connect tubing between the column top and the system valve marked **COLUMN TOP**.
3	Set the system column valves to the ***UpFlow*** position.
4	Using the pump, fill the system with an appropriate liquid for column installation.
5	When the system, including the tubing connected to the column, is filled with liquid, stop the pump.
6	Use the manual valves on the column to change from bypass to in-line.

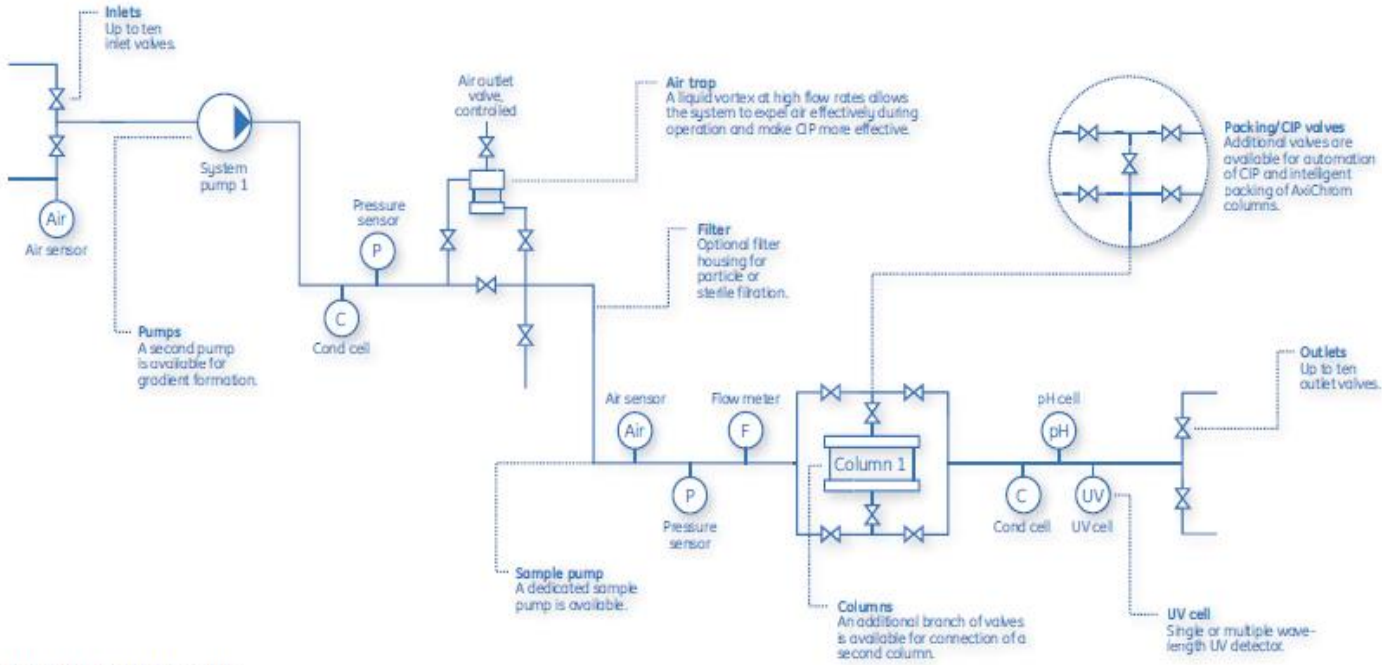


Fig 2. The liquid flow path.

AxiChrom™ 300-1000 columns

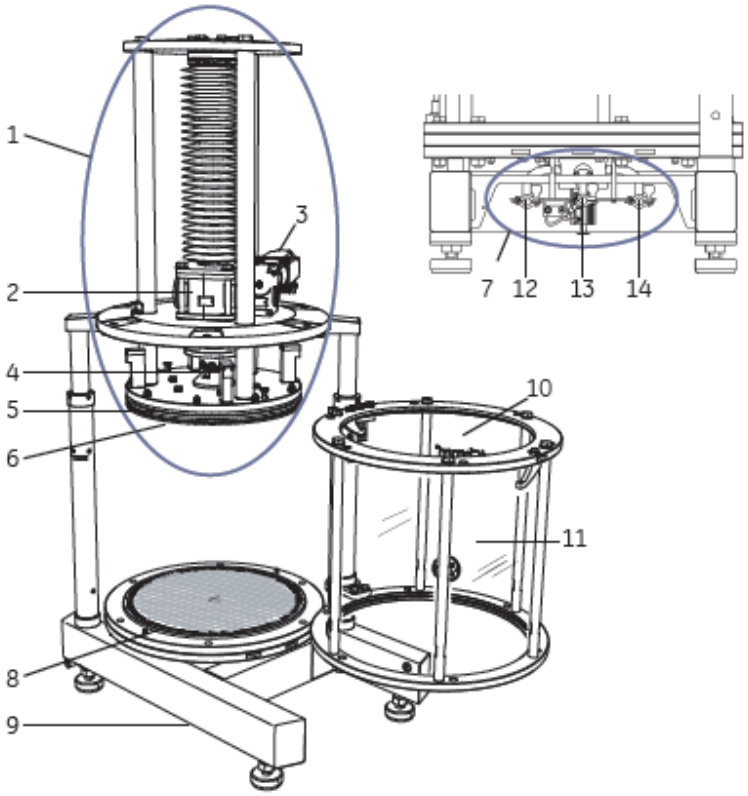
AxiChrom process column family has been designed to deliver reproducible results from process development to production scales. This is facilitated by the innovative Intelligent Packing where UNICORN™ software, ÄKTA™ systems and AxiChrom columns work together to facilitate a convenient operation for packing of the bed via axial compression

The images below show a 600 column (left) and an AxiChrom Master unit (right).

Intended use

The AxiChrom family of process columns has been designed for low pressure chromatographic separation of biomolecules such as proteins, peptides and oligonucleotides in GMP-regulated environments. AxiChrom columns are intended for production use only and should not be used for diagnostic purposes in any clinical or *in vitro* procedures. The columns are not suitable for operation in a potentially explosive atmosphere or for handling flammable liquids. If the columns are used for purposes other than those specified in the user documentation, safe operation and the protection provided by the system may be impaired.

3.1.1.19.Overview of the column and parts



Part	Function	Part	Function
1	Top unit	8	Bottom bed support and distributor plate
2	Worm gear and bellows	9	Column stand
3	Servo motor	10	Process chamber
4	Top mobile phase inlet/outlet	11	Column tube
5	Adapter	12	Slurry inlet
6	Adapter bed support and distributor plate	13	Bottom mobile phase inlet/outlet
7	Media valve assembly	14	Rinse inlet

Inlet and outlet system

- The tubing connection at the Top mobile phase on the adapter is the only liquid connection at the top of the column.
- At the bottom the center inlet/outlet is the bottom mobile phase.
- The Slurry inlet is connected to the slurry tank from where the media slurry is drawn into the column. Inside the Media valve under the column, the Slurry inlet has a liquid connection with the Rinse inlet.
- The Rinse inlet is used to flush the Media valve, the Slurry inlet and the tube to the slurry tank free from residual media after filling and unpacking the column. When a filling process has ended, the Media valve closes and a pump connected to the Rinse inlet pumps the liquid through the Media valve, and then through the Slurry inlet to the slurry tank.

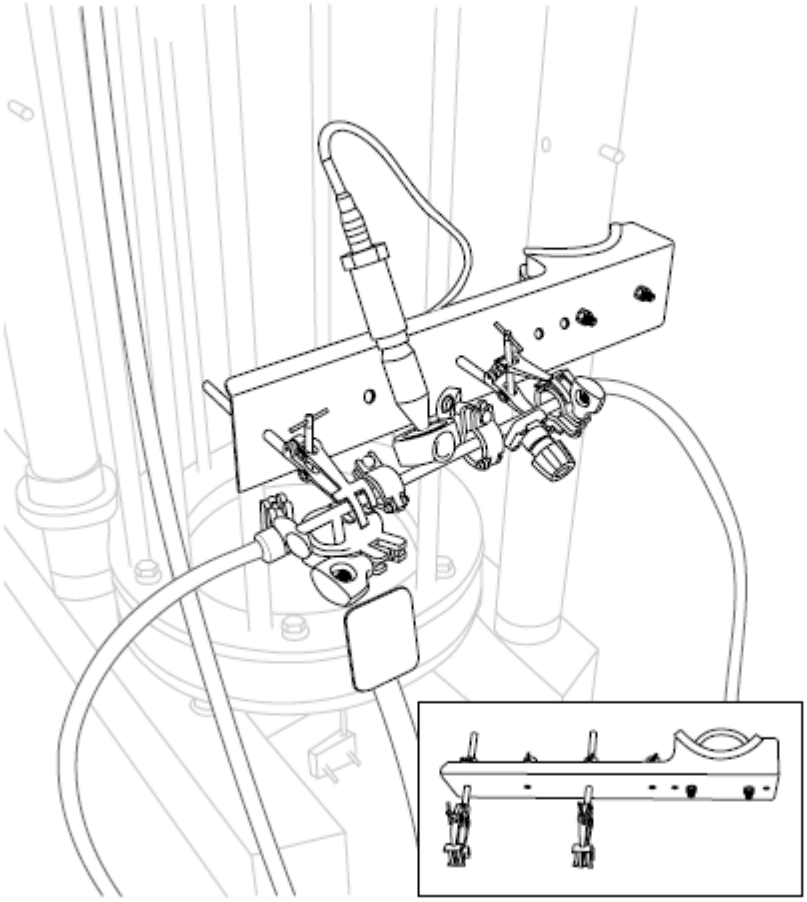
- The Slurry inlet and Rinse inlet have no connection to the Bottom mobile phase flowpath. When the Media valve is open, the Slurry inlet and Rinse inlet lead directly to the process chamber. Liquid in the mobile phases, on the other hand, has to flow through the bed supports to reach the process chamber.

Connection flanges

- **Tri-clamp 25** is used for the 300, 400, 450 and 600 columns.
- **Tri-clamp 50** is used for the 800 and 1000 columns.

Valve holder (accessory)

The valve holder accessory is useful for fixing components to the column assembly, particularly when a number of serially connected valves and sensors are used. The valve holder is clamped on the front stand tube with a U-rod and holds the components with 3-pronged clamps. One valve holder carries two clamps. Different sizes of U-rod are used for AxiChrom 300-600 columns and AxiChrom 800-1000 columns respectively.



Valve holder accessory (inset) and mounted with valves and sensors on front stand tube.

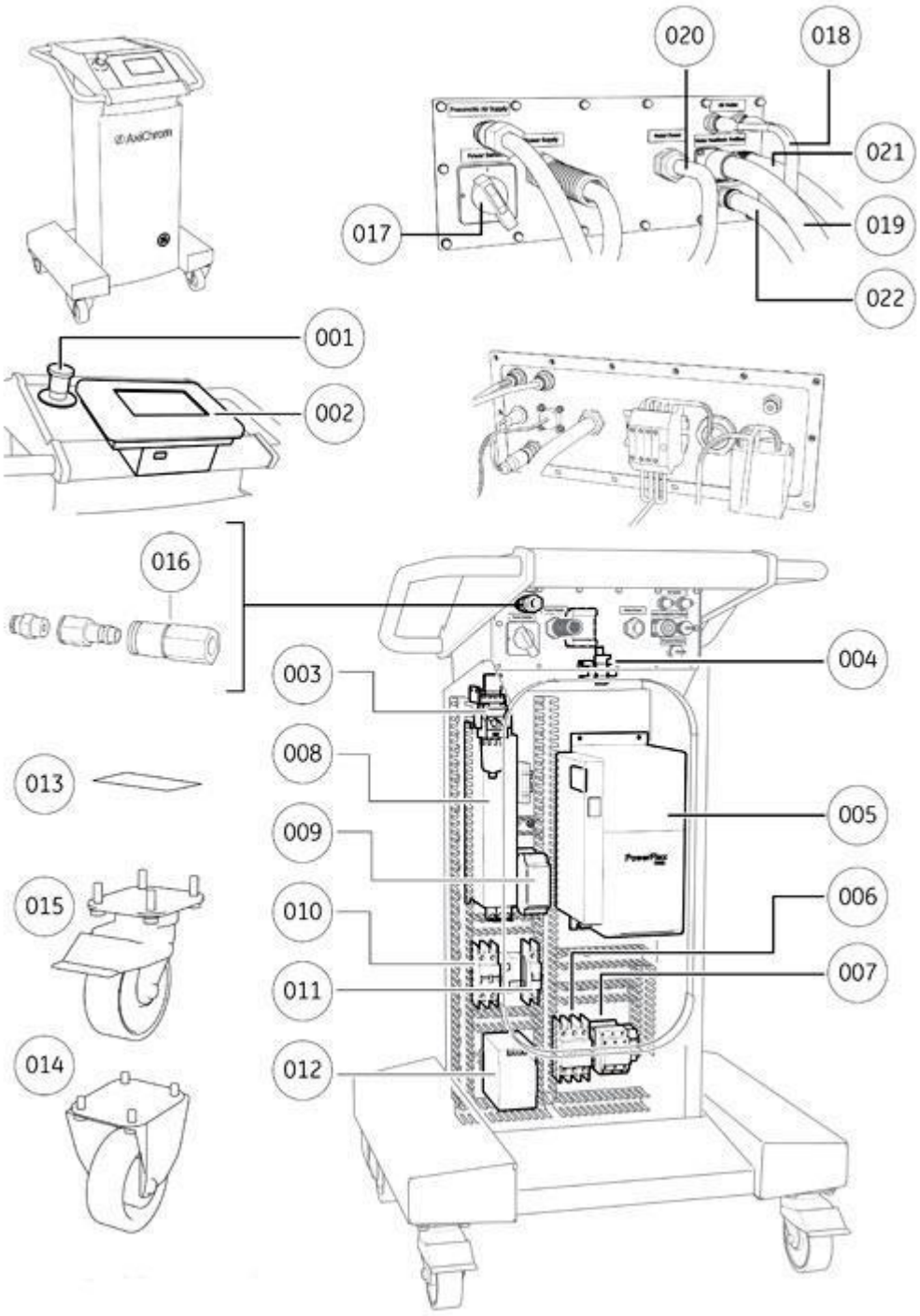
AxiChrom Master

AxiChrom Master is a self-contained operator console featuring interactive guides for work procedures such as packing, unpacking and maintenance. The user interface is a touch screen panel.

The interactive firmware guides the user and reduces the risk of making mistakes. The operator has control over the workflow, and can use manual control for adapter movement and open or close the Media valve.
One AxiChrom Master unit can be used to control up to ten columns (one at a time).

AxiChrom Master specifications

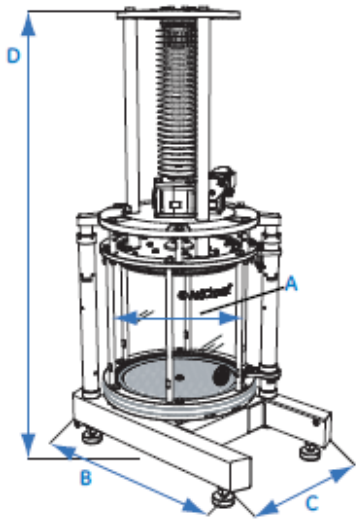
Parameter	Value
Weight	Approximately 73 kg (161 lbs)
Length × width × height	670 × 590 × 1090 mm
Material	Stainless steel, ASTM 316 and ASTM316L (EN 1.4401/1.4436 and EN 1.4404/1.4432/1.4435)



1	Emergency stop switch
2	Display for AxiChrom master
3	Air filter regulator
4	Air solenoid valve
5	AxiChrom Master Control Unit (Spare Part)
6	Circuit breaker

7	Contactor 25A 24V DC
8	Mains filter 3x480 V 16A
9	Safety relay
10	Circuit breaker
11	Circuit breaker 4A, 1-pole
12	Power supply 400 V AC - 24 V DC
13	Wheel gasket
14	Wheel without brake
15	Wheel with brake
16	Air quick connector
17	Mains Switch
18	Air hose kit
19	AxiChrom master encoder cable
20	AxiChrom master motor cable
21	AxiChrom master profibus cable
22	AxiChrom master pressure cable

Weights, volumes and related dimensions of axichrom



Column	300	400	450	600	800	1000
Tube inner diameter (A) (mm)	300	400	450	600	800	1000
Footprint (B×C) (mm)	1110 × 520	1110 × 600	1110 × 620	1180 × 780	1470 × 1080	1720 × 1300
Safety zone (mm)	3200 × 2600	3200 × 2600	3200 × 2700	3200 × 2800	3500 × 3100	3800 × 3300
Column cross section (cm^2)	707	1257	1590	2827	5027	7854
Max bed volume, short/long tube (liters)	21/35	36/63	48/80	85/141	151/251	236/393
Weight of empty column, short/long column, stainless steel bed support (kg)	420/440	460/480	710/760	835/900	2150/2240	2560/2680
Weight of empty column, short/long column, plastic bed support (kg)	414/434	451/471	699/749	818/883	2122/2212	2517/2637

Heights in different operating states

Column	Tube	300	400	450	600	800	1000
Max height [1] (D)	Short [2]	2200		2230	2340	2630	2650
	Long [3]	2720		2750	2860	3150	3170
Height (D) when adapter is at max bed height	Short	1740		1760	1870	2160	2170
	Long	2200		2220	2330	2620	2630
Min height (delivery height)	Short	1450	1460	1480	1590	1880	1890
	Long	1710	1720	1740	1850	2140	2150
Max slurry filling height	Short	2040		2060	2185	2335	2490
	Long	2555		2580	2700	2850	3005
Max operating height (D) during priming	Short	2060		2080	2190	2480	2490
	Long	2580		2600	2710	3000	3010
Max adapter stroke height for filling	Short	570				578	
	Long	830				838	

Operating conditions

Parameter	Value
Maximum operating pressure [1]	4 bar
Operating temperature [2]	2°C to +30°C
Operating pH [3]	1 to 14

3.1.1.20.Materials

Background information

The materials used to manufacture AxiChrom columns have been chosen for their biological and chemical compatibility with the solvents used during operation and cleaningin-place (CIP) procedures. The columns have also been designed to comply with the varying hygienic requirements at the different stages of development and production. Polymer materials in AxiChrom columns in contact with process liquids have been selected for their biological compatibility according to the United States Pharmacopeia (USP) Biological Reactivity Tests, *In vivo* and conform to USP class VI requirements, compliance with Code of Federal Regulations (CFR), Food and Drug Administration, Title 21, Part 177 and being animal free or complies with the conditions in the CPMP Note for Guidance (EMEA/410/01 Rev.2).

Column tubes for AxiChrom 300-1000 columns are available in acrylic plastic or stainless steel. Use and maintenance of AxiChrom columns with stainless steel column tubes differs from that for acrylic plastic column tubes in two major respects:

- Stainless steel column tubes are not transparent, so the bed cannot be observed directly. This affects packing and unpacking procedures.
- Stainless steel column tubes are manufactured in a single piece, with no removable tie rods, top or bottom flanges or corresponding O-rings. This does not affect normal operation or maintenance procedures but results in a spare parts and accessories list that differs from that of acrylic columns.

Parts list and materials

Component	Material	In contact with process stream
Adapter backing plate	Stainless steel ASTM 316 or ASTM S32205	No
Adapter seals and snap ring	UHMWPE (ultra high molecular weight polyethylene)	Yes

Component	Material	In contact with process stream
Bed support	Stainless steel ASTM 316L and ASTM S32205 or PE (polyethylene) or UHMWPE	Yes
Column tube	PMMA (polymethyl methacrylate) or Stainless steel ASTM 316L	Yes
Distributor	PP (polypropylene)	Yes
Dynamic seals	FFPM (full fluorinated propylene monomer) or UHMWPE	Yes
Media valve body	PP	Yes
Static seals	EPDM (ethylene propylene diene monomer)	Yes
Top mobile phase	PP	Yes
Bottom backing plate	Stainless steel ASTM 316	No
Lid	Stainless steel ASTM 316	No
Stand	Stainless steel ASTM 316	No

Chemical resistance

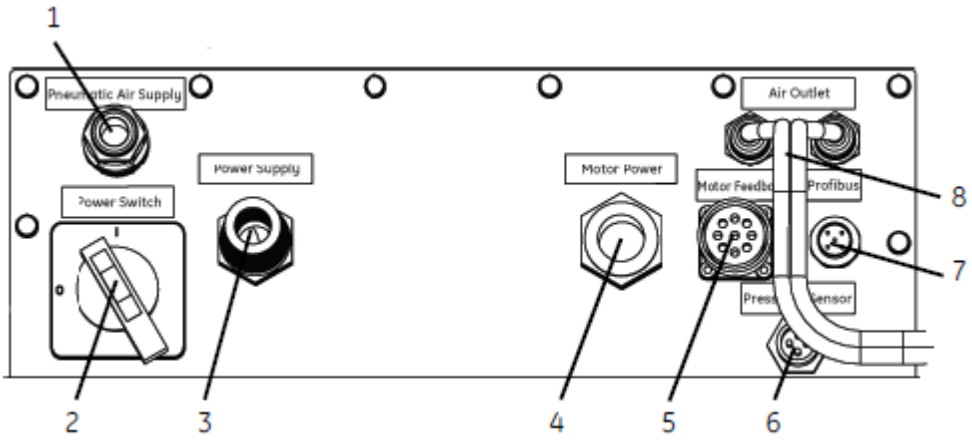
AxiChrom columns are resistant to chemical agents used in protein recovery, including buffer solutions for adsorption, elution and washing, and to solutions effective in cleaning, sanitization and storage. *Table* lists chemicals that may or may not be used with AxiChrom columns. The concentrations listed are not normally exceeded during an operating cycle.

Chemical	Concentration [1]	Time/cycle restrictions	Comments	Operating temperature	CAS no. [2]
Acetic acid	25%	3 h	Cleaning-In-Place (CIP)	2°C to 30°C	64-19-7
Acetone	2%	1 h	Efficiency test	2°C to 30°C	67-64-1
Ammonium sulphate	2 M [3]	5 h	Adsorption	2°C to 30°C	7783-20-2
Benzyl alcohol	2%	12 months	Storage	2°C to 30°C	100-51-6
Ethanol	20%	12 months and max. 0.5 bar	Storage	2°C to 30°C	64-17-5
Ethanol	70% [4]	3 h	CIP	2°C to 30°C	64-17-5
Ethanol/ acetic acid	20%/ 10%	3 h	CIP	2°C to 30°C	64-17-5/ 64-19-7
Guanidinium hydrochloride	6 M [5]	5 h	CIP	2°C to 30°C	50-01-1
Hydrochloric acid	0.1 M (pH = 1) [6]	1 h	CIP	2°C to 30°C	7647-01-0
Isopropanol	30% [7]	1 h	CIP	2°C to 30°C	67-63-0
Phosphoric acid	5%	8 h	For passivation of stainless steel bed supports	2°C to 30°C	7664-38-2
Sodium chloride	0 to 3 M [3, 6, 8]	3 h	Purification, CIP	2°C to 30°C	7647-14-5
Sodium hydroxide	1 M (pH = 14)	24 h, room temp. to 30°C	CIP	2°C to 30°C	1310-73-2
Sodium hydroxide	0.01 M (pH = 12)	12 months	Storage	2°C to 30°C	1310-73-2
Sodium hydroxide/ ethanol	1 M/ 20%	3 h	CIP	2°C to 30°C	1310-73-2/ 64-17-5
Sodium sulphate	1 M [3]	3 h	Adsorption	2°C to 30°C	7757-82-6
Urea	8 M [3]	5 h	Purification, CIP	2°C to 30°C	57-13-6
Commonly used aqueous buffers for chromatographic use	10 to 250 mM, pH 2 to 10	24 h	Equilibration, adsorption, elution	2°C to 30°C	

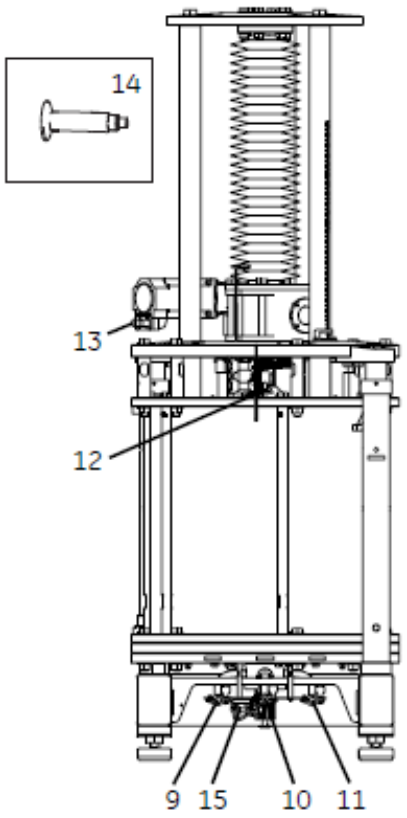
3.1.1.21.Connections

Connections on Master and column

The illustrations below show the AxiChrom Master connector panel and the connection points on a column.



Part	Description	Part	Description
1	Pneumatic air supply	5	Motor feedback
2	Power switch	6	Pressure sensor connector
3	Power supply cable	7	Profibus signal cable connector
4	Motor power cable	8	Two air outlet connectors



Part	Description
9	Slurry inlet
10	Bottom mobile phase inlet/outlet
11	Rinse inlet
12	Top mobile phase inlet/outlet
13	Motor power and motor feedback
14	Pressure sensor (PIS_119, mounted on hose to system)
15	Pneumatic inlets with 2 connectors

Connect column, Master, and external system

The table below shows how to connect the column, Master and an external system.

From	To
Pneumatic air supply (1) on the Master	**Wall socket air outlet** (5.5-7 bar)
Motor power cable (4) on the Master	**Motor power** (the right connector) on the column (13)
Motor feedback (5) on the Master	**Motor feedback** (the left connector) (13) on the column
Pressure sensor connector (6) on the Master	**Pressure sensor** mounted on system (14)
Profibus signal cable (7) on the Master	Profibus connection on an ÄKTAprocess system
Two air outlet connectors (8) on the Master	**Pneumatic inlets with 2 valves** (15) on the column
Slurry inlet (9) on the column	**Slurry tank**
Bottom mobile phase (10) on the column	**Bottom mobile phase** on a system (**Column1 bottom valve** on the ÄKTAprocess system)
Rinse inlet (11) on the column	A system (**CIP2 Inlet** on the ÄKTAprocess system)
Top mobile phase (12) on the column	**Mobile phase** on a system (**CIP1 Inlet** on the ÄKTAprocess system)
Protection ground cable on the column stand	**Ground** (See *Grounding the column, on page 65*)
Power supply cable (3) on the Master	**Power supply connector** (380-400 VAC, 50-60 Hz) with protective ground (The AxiChrom Master is delivered with CE or UL approved cables.)

Recommended mobile phase tubing inner diameters

Note: *• All dimensions are given in millimeters.*

• A dash (-) means that the combination is not compatible with Intelligent Packing.

• TC25 connectors do not have the same inner diameters as the tubing. See Tubings, on page 187 for details.

Table 3.1: Tubing inner diameters recommended for top and bottom mobile phase connections for different column inner diameters.

ÄKTAprocess dimension	300	400	450	600	800	1000
6 mm PP	6.4	6.4	6.4	-	-	-
	9.4	9.4	9.4			
3/8" SS	6.4	6.4	6.4	-	-	-
	9.4	9.4	9.4			
10 mm PP	6.4	6.4	6.4	9.4	-	-
	9.4	9.4	9.4	12.7		
	12.7	12.7	12.7	19.1		
1/2" SS	6.4	6.4	6.4	9.4	-	-
	9.4	9.4	9.4	12.7		
	12.7	12.7	12.7	19.1		
1" PP and SS	-	-	-	9.4	25.4	25.4
				12.7	34.7	34.7
				19.1		

Power requirements and connections

The general requirements are:

Requirement	Value
Supply voltage	380-400 VAC
Nominal current	10-15/16 A NTD (Non-Time Delay) (min. -max.)
Frequency	50 - 60 Hz
Max voltage (North America)	480 Y/277 VAC
Max current	6 A
Max power consumption	2400 VA
Short circuit rating	5 kA

Color coding of cable conductors

Conductor	Color
Protective ground (earth)	Green/yellow
Live 1	No 1 or Black
Live 2	No 2 or Brown
Live 3	No 3 or Grey

The black, brown and grey conductors may be connected to any of Live 1, 2 and 3. The phase connection is detected automatically

. Compressed air requirements

It is important for personal safety and safe operation to use the correct pressure and quality of compressed air for the pneumatic valve control. The basic requirements are:

- Free of oil and particles
- -30ºC dew point
- 5.5 to 7 bar

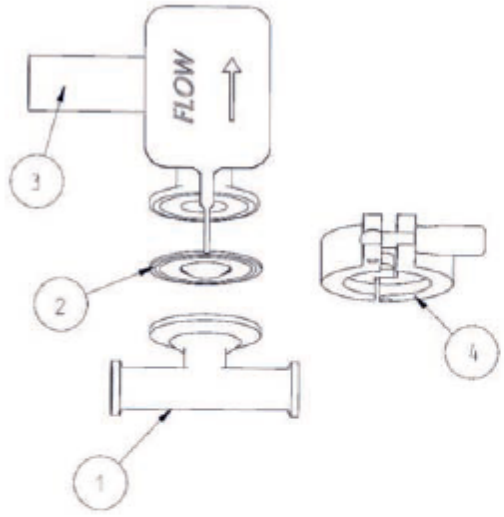
The pneumatic air supply connections on AxiChrom Master are illustrated in *Connections on Master and column, on page 59*.

Rupture discs

To secure equipment and personal safety, rupture discs are available as accessories. *Installation of rupture discs will change the maximum operating pressure for the column from 4 to 3.8 bar (g) due to rupture disc characteristics.*

Note:

For column sizes 300-600 there is a special T-junction provided for use with rupture discs.



Part	Description	Part	Description
1	T-junction	3	Vent
2	Rupture disc	4	Clamp

Figure 3.2: Rupture disc assembly

3.1.1.22.Column and system setup

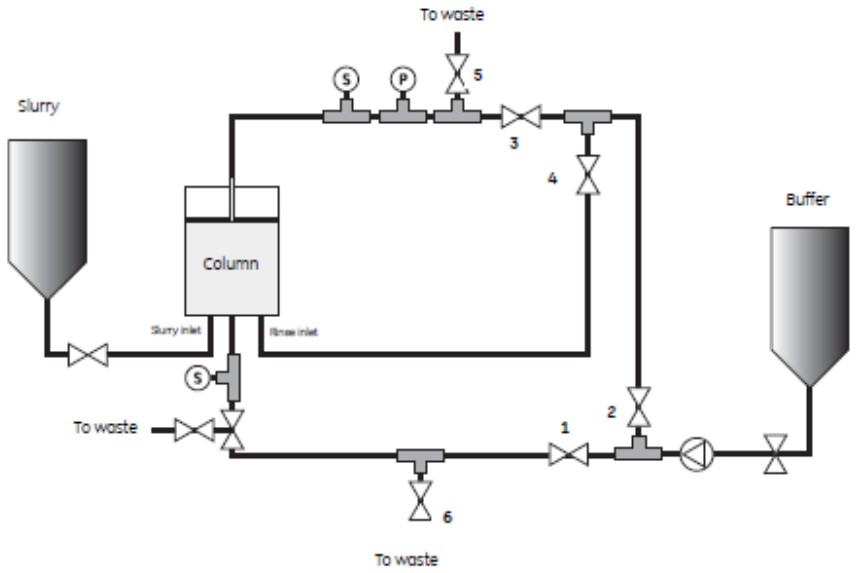
In this section

This section describes how to connect the AxiChrom column to tanks, pumps and for example an ÄKTAprocess system, for priming, packing and unpacking sequences in the

AxiChrom Master.

Manual configuration

The following manual setup makes it possible to perform all processes available in the AxiChrom Master: ***PRIMING***, ***INTELLIGENT PACKING*** and ***UNPACKING***. The setup comprises two way valves and shut-off valves to utilize different flow paths for the different procedures.



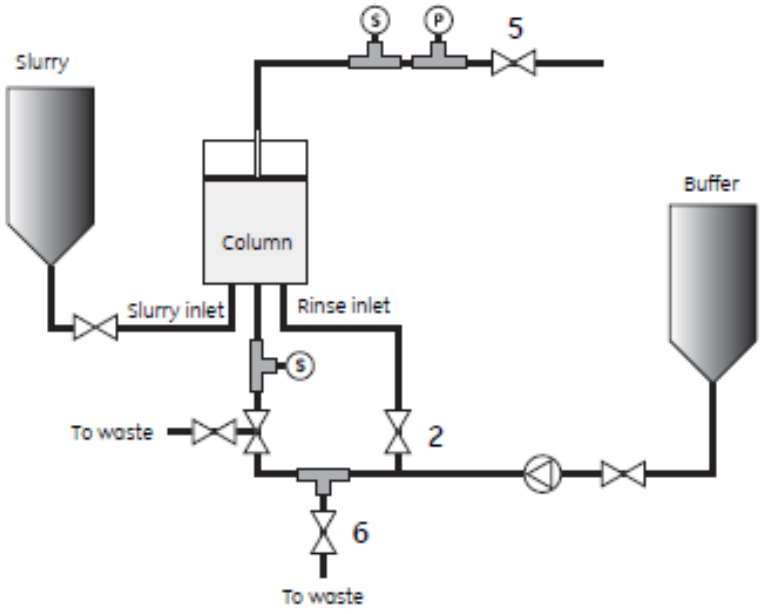
Symbol	Function
	Pressure relief valve, rupture disc or similar
	T-piece
	2-way membrane valve/shutoff valve
	2-way membrane valve/shutoff valve with bleed, can be replaced with two 2-way membrane valves and a T-piece

Possible flowpaths for different column operations

Process	Flow path alternatives (from > to)
Priming	Pump > bottom mobile phase > waste (valve 5)
	Column > bottom mobile phase > waste (valve 6)
Packing	Column > bottom mobile phase > waste (valve 6)
Rinse	pump > valve 2 > valve 4 > rinse > slurry tank
Unpacking "upflow"	pump > bottom mobile phase > top mobile phase > waste (valve 6)
Unpacking "media push out and down flow"	pump > bottom mobile phase > top mobile phase > closed > media valve open to slurry tank
	pump > valve 2 > top mobile phase > bottom mobile phase > waste (valve 6)

Manual configuration: minimal setup

The following manual setup makes it possible to perform the ***PRIMING*** and ***INTELLIGENT PACKING*** procedures available in the AxiChrom Master. The setup comprises two way valves and shut-off valves to utilize different flow paths for the different procedures.

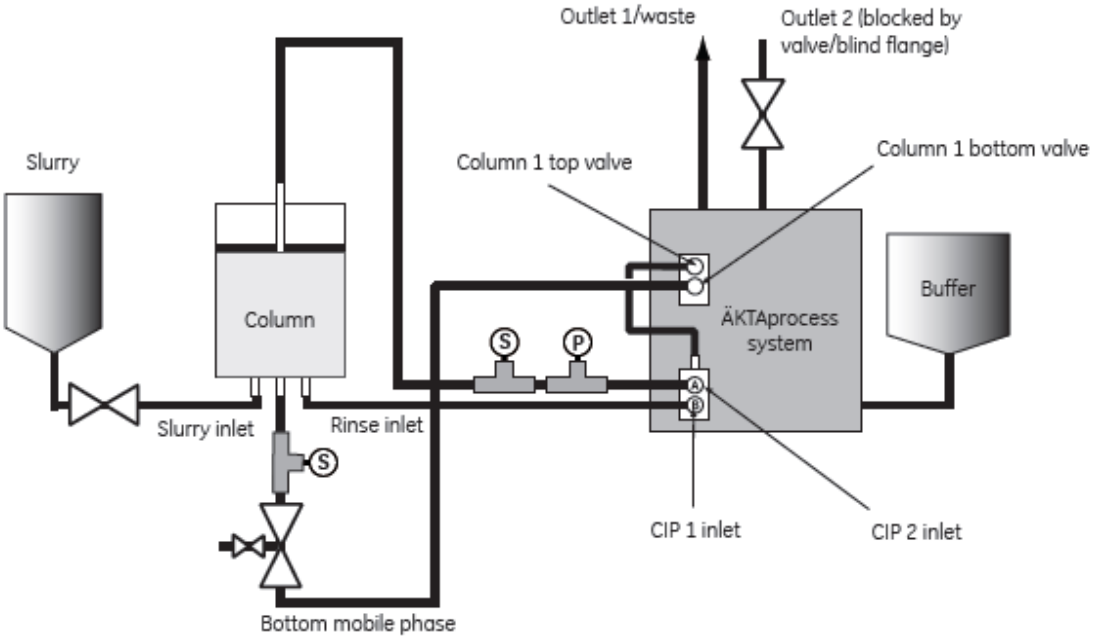


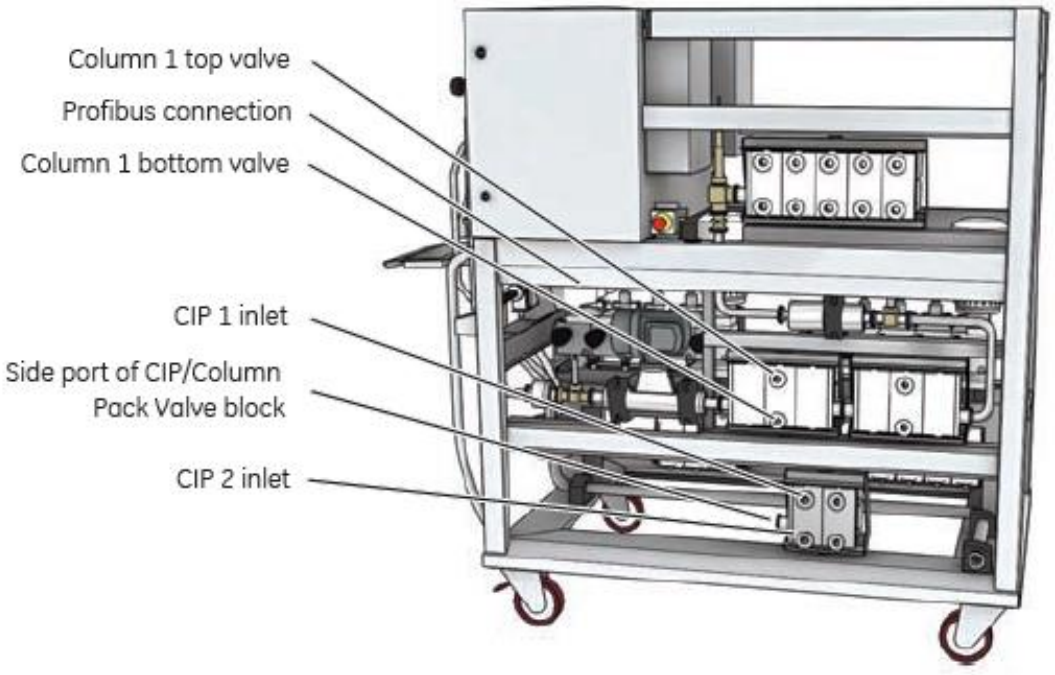
Possible flowpaths for different column operations

Process	Flow path alternatives (from > to)
Priming	Pump > bottom mobile phase > top mobile phase > waste (valve 5)
	Column > bottom mobile phase > waste (valve 6)
Packing	Column > bottom mobile phase > waste (valve 6)
Rinse	Pump > valve 2 > rinse > slurry tank

Automatic configuration connecting ÄKTAprocess

The following setup makes it possible to perform all procedures that are available in the Method wizard in UNICORN: ***Priming***, ***Intelligent Packing*** and ***Unpacking***. The setup is simplified by using ÄKTAprocess controlling the pump and valves for flow directions.





Connecting to an ÄKTAprocess system

To simplify automated priming, column packing with HETP evaluation or column unpacking procedure, the AxiChrom column has to be connected to a ÄKTAprocess system (equipped with the optional CIP/Column Packing valve block) as described below.

Procedure

1 Connect a short piece of tubing of suitable length and the same inner diameter as the system, from **Column1 top valve** to **Side port of the CIP/Column Pack valve block**.

2 Connect the AxiChrom Master pressure transmitter **PIS_119** to **INLET CIP1**.

3 Connect a pressure relief valve or similar to **PIS_119**.

4 Connect a tube of suitable length and inner diameter, from the column **Top mobile phase** to the pressure relief valve on AxiChrom Master pressure transmitter **PIS_119**. For tube diameter recommendations see *Recommended mobile phase tubing inner diameters, on page 62*.

5 Connect a tube of suitable length and inner diameter from **CIP2** on the process system CIP/Column Pack valve block to the Rinse inlet on the column.

6 Connect a tube of suitable length and inner diameter from **Bottom mobile phase** on the column to a pressure relief valve, rupture disc or similar and then to **Column1 bottom valve** on the process system.

7 Connect the profibus cable from the Profibus connection situated under the

electrical cabinet of the ÄKTAprocess system to the Profibus connection on the AxiChrom Master.

Connecting AxiChrom Master to ÄKTAprocess via Profibus

Connect the Profibus cable

1 Power down the ÄKTAprocess and Master units.

2 Remove the Profibus termination plug from the ÄKTAprocess bulkhead connector.

3 Connect the Profibus cable to the ÄKTAprocess bulkhead connector. Make sure that the cable barrel is straight and engages correctly in the threads of the connector.

4 Remove the protective cap from the Profibus connector of the Master unit.

5 Connect the Profibus cable to the Profibus connector of the Master unit.

3.1.1.23.Appendix A Parts list and diagrams

A.1 Part numbers and names

Position	Name
104	Tube holder 400 to 1000
105	Tube holder bracket
106	Holder
109	Locking part
111	Fastener, adaptor
115	Fastener, Tube holder 300 to 600
116	Flushing connection
117	Flushing connection nut
126	Plate
127	Shock absorber, lower
128	Top plate
129	Level display
132	Tube Roller, asm
133	Flexible tube 3/4in, TC25
134	Lid support, complete
136	Locking pin, asm
137	Flushing Inlet Tubing L1000
143	Hex Head Screw ISO 4017 M6x25 A4-70
144	Screw M6S-H M5X14

Position	Name
145	Screw M10 X 25
146	Hex Head Screw ISO 4017 M12x45 A4-70
148	Hex Head Screw ISO 4017 M16x40 A4-70
149	Hex Head Screw ISO 4017 M16x45 A4-70
150	Hex Head Screw M12x45
151	Hex Head Screw M12x45
152	Hex Head Screw M12x45
154	Parallel Pin ISO 2338 12m6x60 A2
155	Foot M30
157	Hex Nut ISO 4032 M30 A4-70
159	Domed Cap Nut M10
160	Hex Head Screw ISO 4017 M6x16 A4-70
161	Washer ISO 7089 6.4x12x1.6 A4
163	Washer ISO 7089 13x24x2.5 A4
165	Washer 21x37
169	Washer Nylon 13x24x2.5
170	Washer Nylon 10.5x21x2.5
171	Motor
172	Hex Head Screw ISO 4017 M24x60 A4-70
173	M6S M20x200 A4-70
174	Hex Head Bolt ISO 4014 M16x80 A4-70
175	Hex Socket Head Cap Screw ISO 4762 M10x30 A4-70
178	Hex Socket Head Cap Screw ISO 4762 M8x30 A4-70
181	Hex Nut ISO 4032 M16 A4-70
182	Hex Nut ISO 4032 M8 A4-70
193	Pneumatic Connection Plug
194	Pneumatic Connection Plug

Position	Name
196	O-ring 54x3
197	O-ring 6.02x2.62
198	O-ring 26.64x2.62
201	Column tube
202	Tie rod
203	Flange, bottom
204	Flange, top
211	O-ring 673.1x9.525
213	Hex Head Screw M20x60
221	Hinge lower asm
231	Hex head screw ISO 4017 M16x45 A4-70 (SSl column tube)
232	Hex head screw ISO 4017 M16x50 A4-70 (SS column tube)
301	Bed support bottom 600, 10 or 20 µm
302	Bed support adapter 600, 10 or 20 µm
401	Distributor bottom 600
402	Distributor adaptor 600
403	Bed support screw bottom 300-600
404	Bed support screw adaptor 300-600
405	Distributor adaptor ring 600
406	Snap ring 600
409	Fastener, bottom
411	T-piece, 3TC, ASME BPE DT- 18, 1/2in, SF5
412	Pressure Transmitter
413	O-ring 26.7x1.78
414	O-ring 600x10
415	O-ring 556.86x6.985
416	O-ring 506.86x6.985

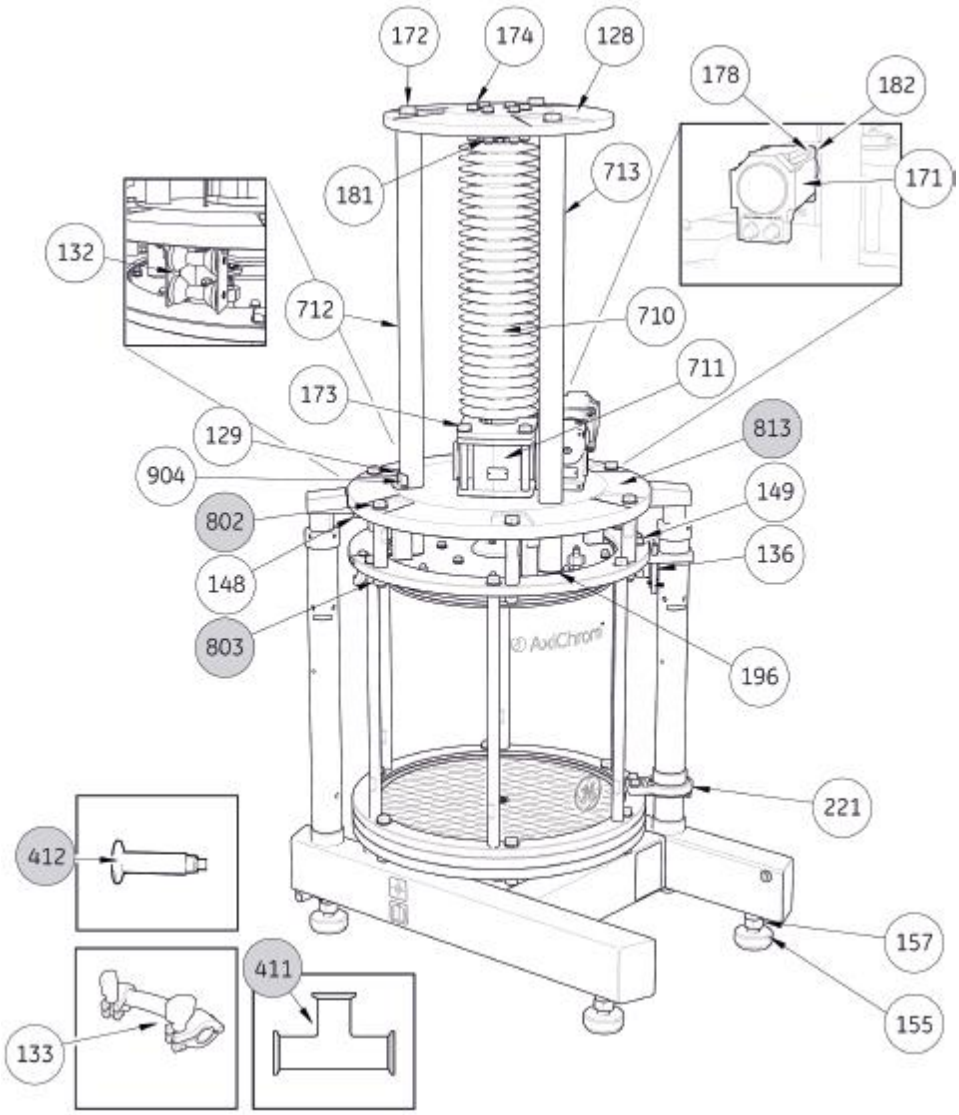
Position	Name
417	O-ring 30x3.5
421	Hex Socket Head Cap Screw ISO 4762 M8x25 A4-70
422	Washer ISO 7089 8.4x16x1.6 A4
423	Parallel Pin ISO 2338 6m6x16 A2
431	Hex Nut ISO 4032 M10 A4-70
432	Washer ISO 7089 10,5x20x2 A4
501	Tube Mobile Phase, bottom
502	Tube Slurry inlet/outlet, bottom
503	Tube Rinse, bottom
504	Connector top inlet
505	End cap
506	TC clamp for TC 25
507	TC-gasket, TC25 ID15, EPDM
508	Inner Connector Rinse (only used with PP tubes on 300-600 columns)
509	O-ring (only used with PP tubes on 300-600 columns)
511	Top Inlet/outer body
512	O-ring 16x3 EPDM70
513	Parallel Pin ISO 2338 4M6x16 A2
516	Valve body, outer
517	Valve body, inner
518	O-ring holder
519	Piston
521	Pneumatic cylinder
537	Washer
541	O-ring 125x5
542	Top inlet
543	Valve

Position	Name
601	O-ring 14x3
602	O-ring 48x3.5
603	O-ring 21.2x3
604	O-ring 27x4
607	O-ring 48x3.5
611	O-ring 48x3.5
612	O-ring 27x4
614	O-ring 48x3.5
616	O-ring 14x1.78
617	O-ring 14x1.78
618	O-ring 8x3
624	O-ring 14x3
628	O-ring 565x11
641	Scraper
710	Bellows
711	Screw and gear
712	Adapter rod with level scale
713	Adapter rod
801	Hex Head Bolt M20×110
802	Hex Head Bolt M24×300
803	Hex Nut M24
811	Bottom backing plate
812	Adapter backing plate
813	Lid
814	Adapter stop
904	Level display label

3.1.1.24.A.2 Columns with any bed support material

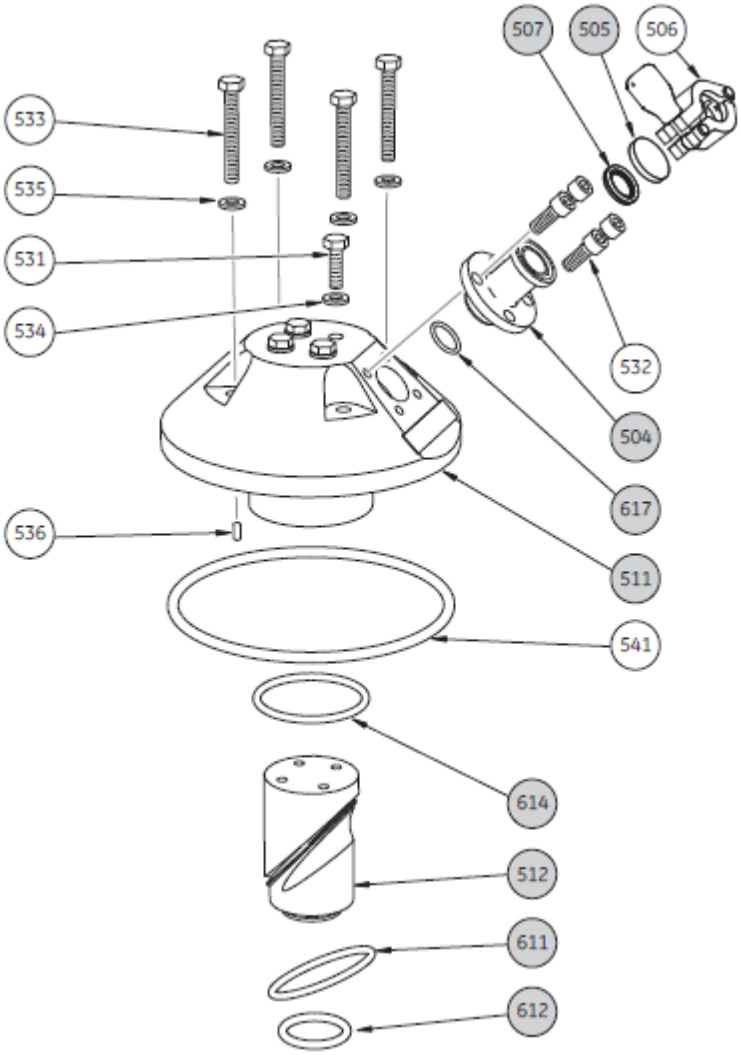
Note: • *The numbers correspond to position numbers in Material Conformity and Spare Parts.*

• Grey circles indicate parts which retain pressure or are in contact with process fluids



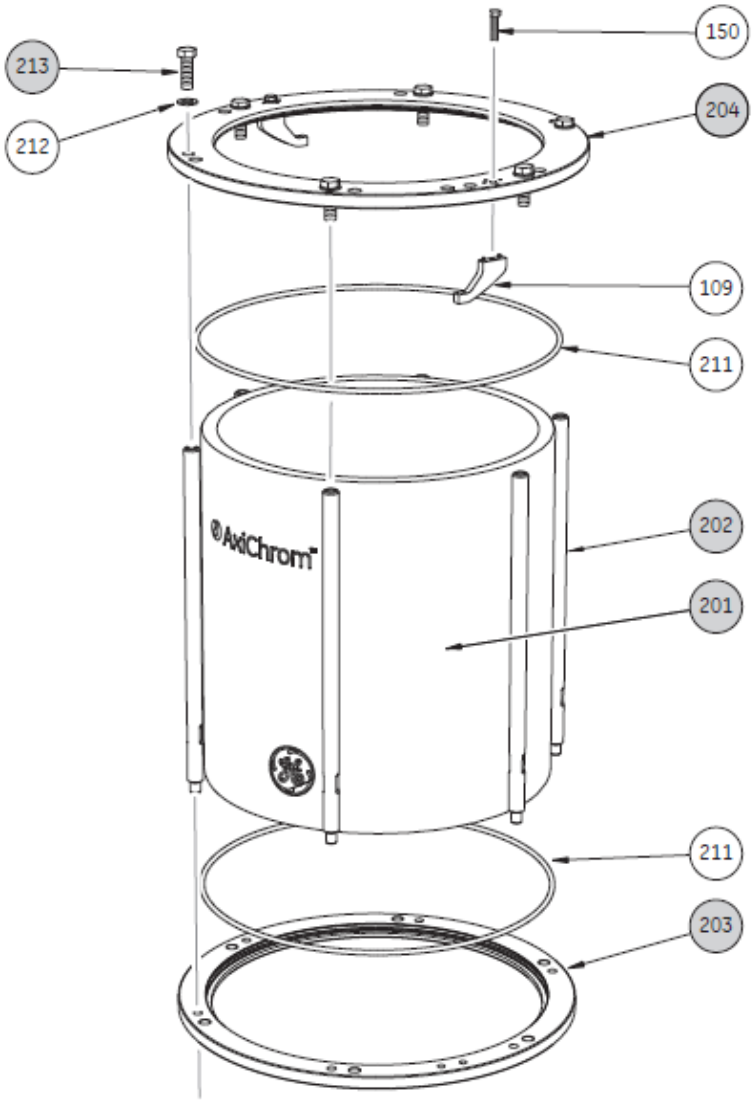
Parts 411 and 412 are mounted on the system.

Part 133 (Storage solution fill hose) is dismounted after commissioning

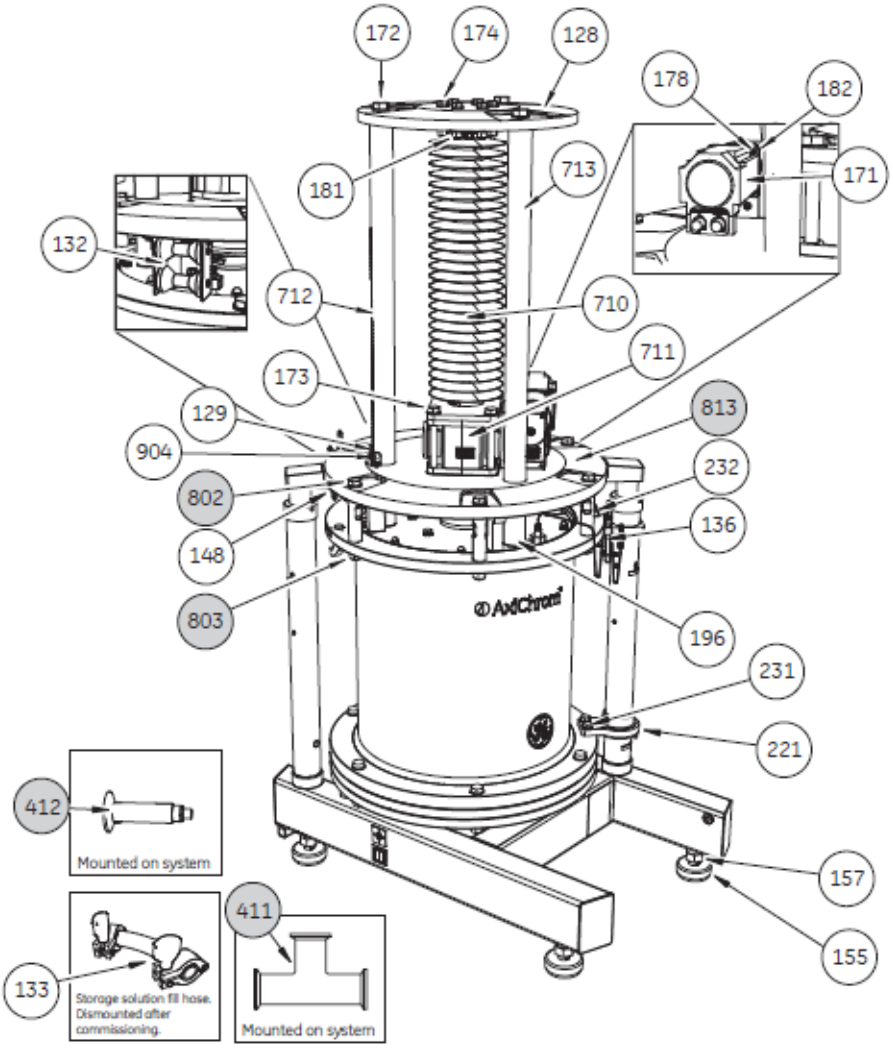


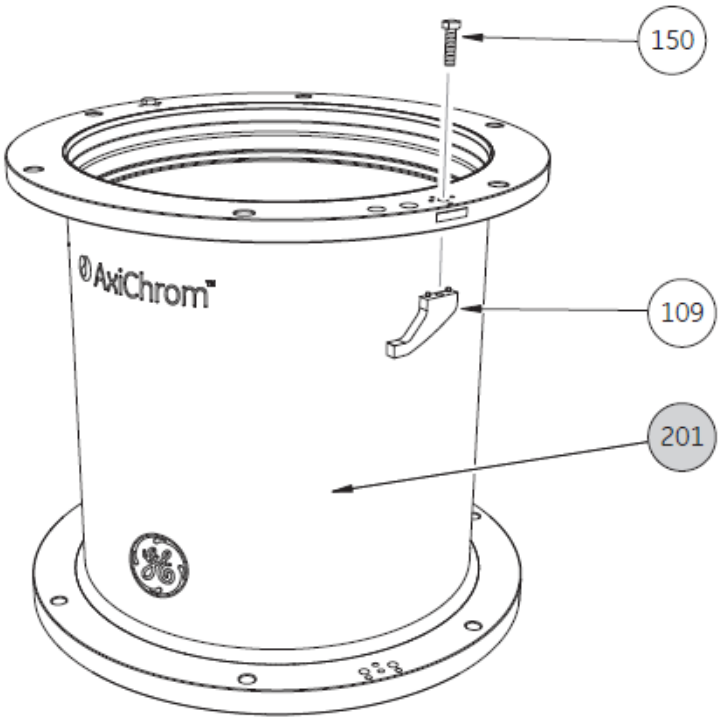
Part 536 is not included in newer inlet/outlets.

11.4.3 Columns with acrylic tubes



3.1.1.25.Columns with stainless steel tubes




150
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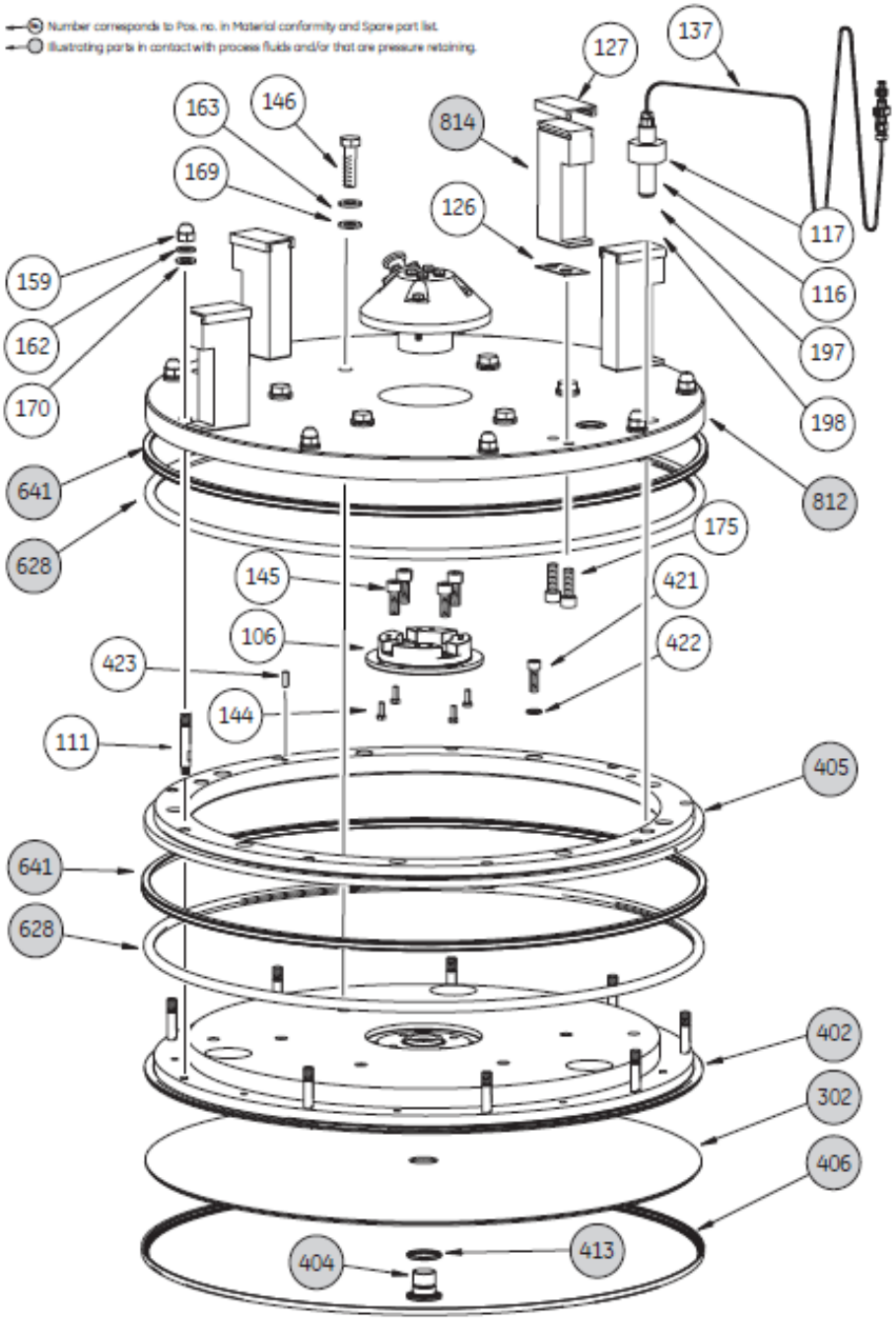
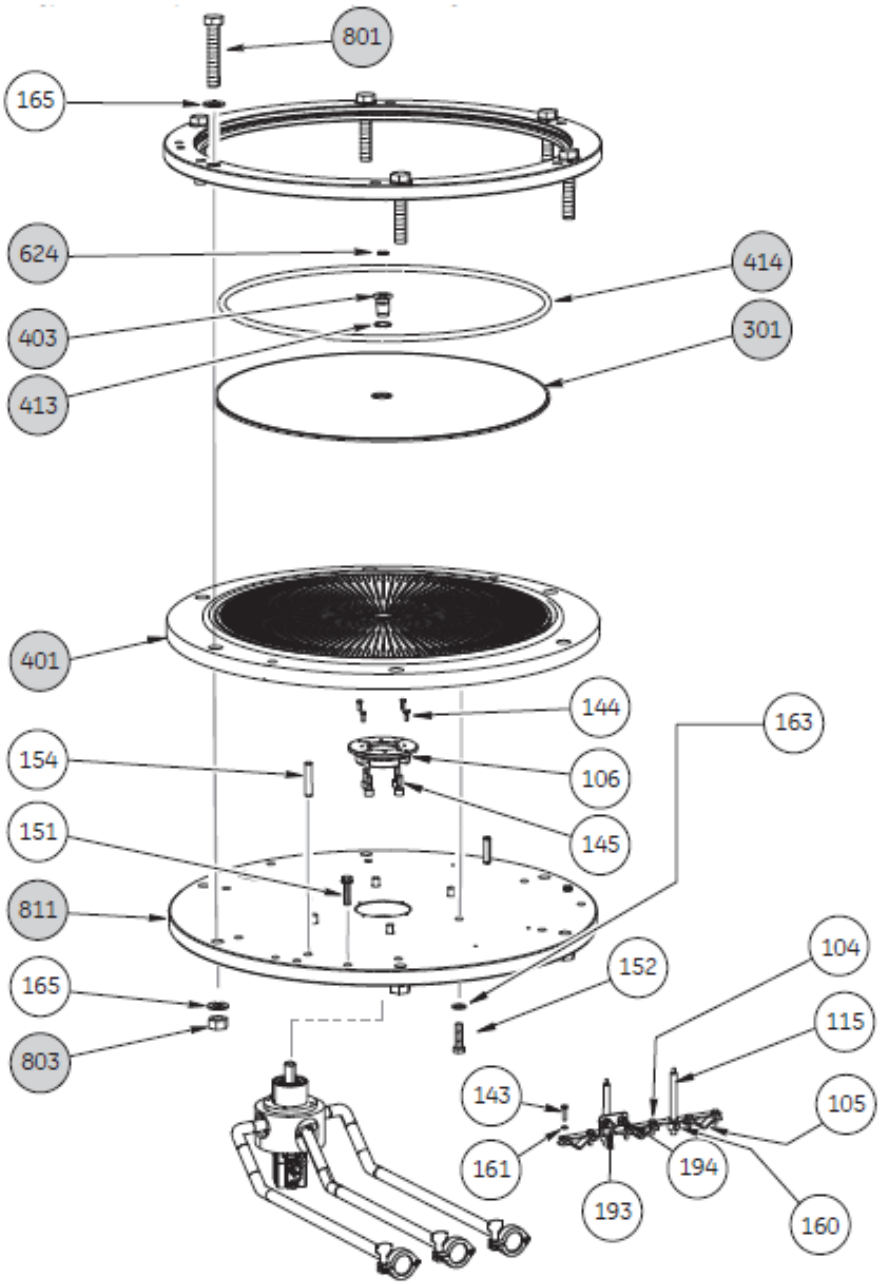
3.1.1.26.A.3 Columns with plastic bed supports

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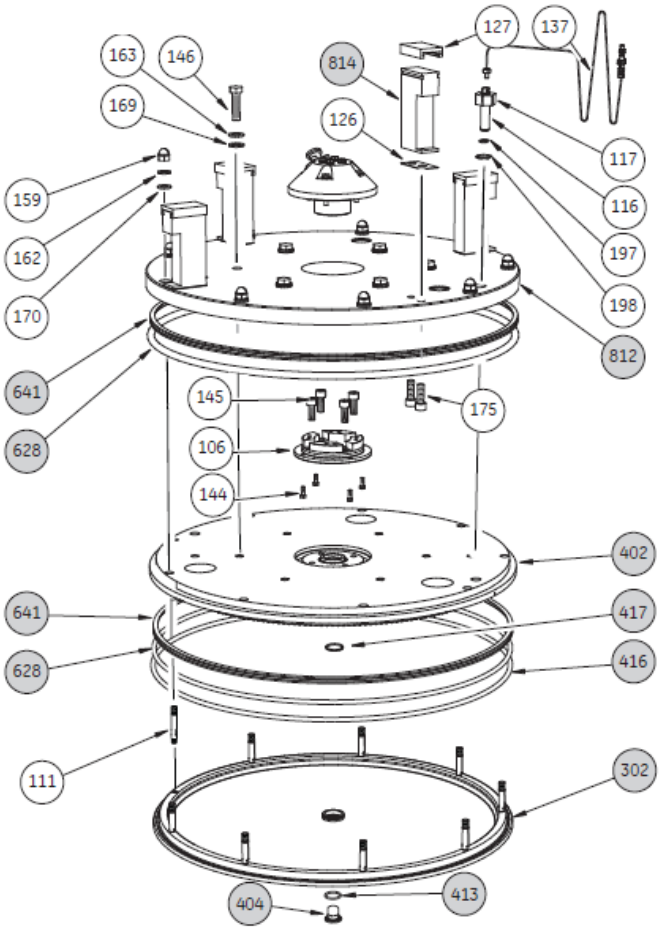
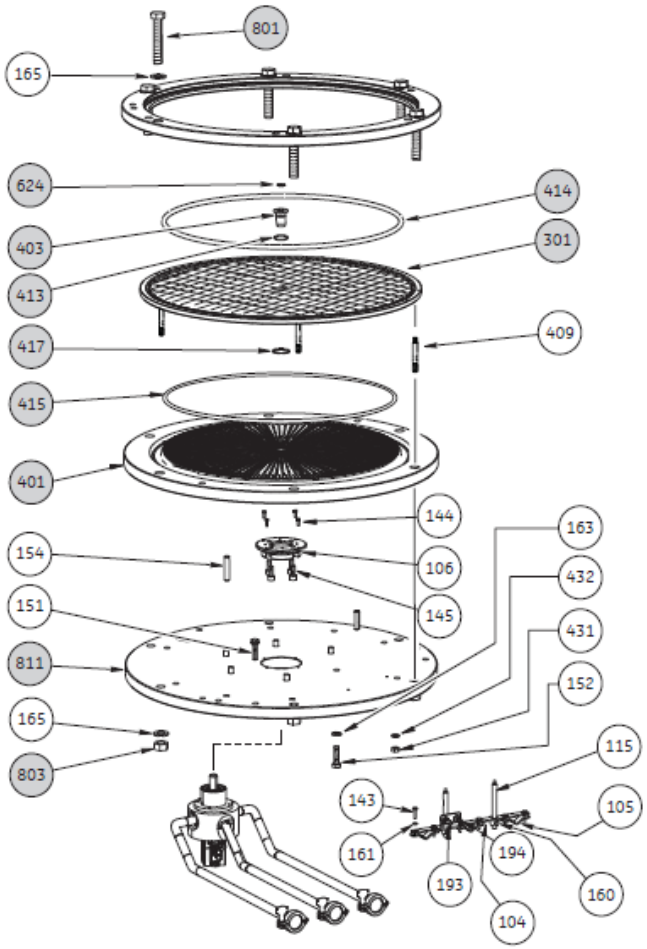
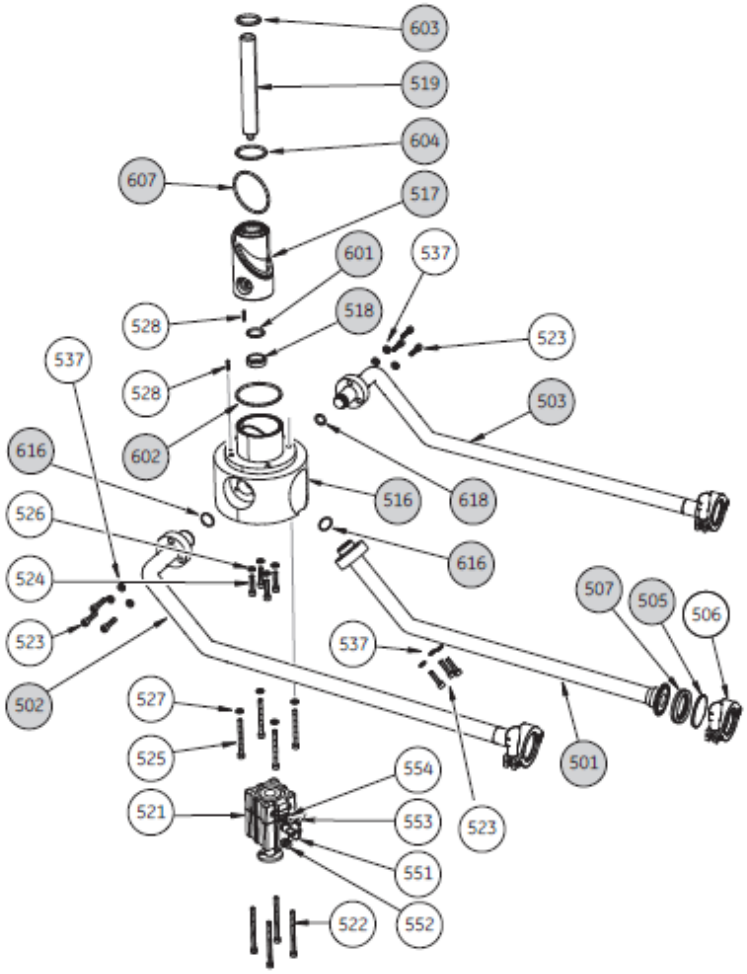
11.4.4 A.4 Columns with stainless steel bed supports



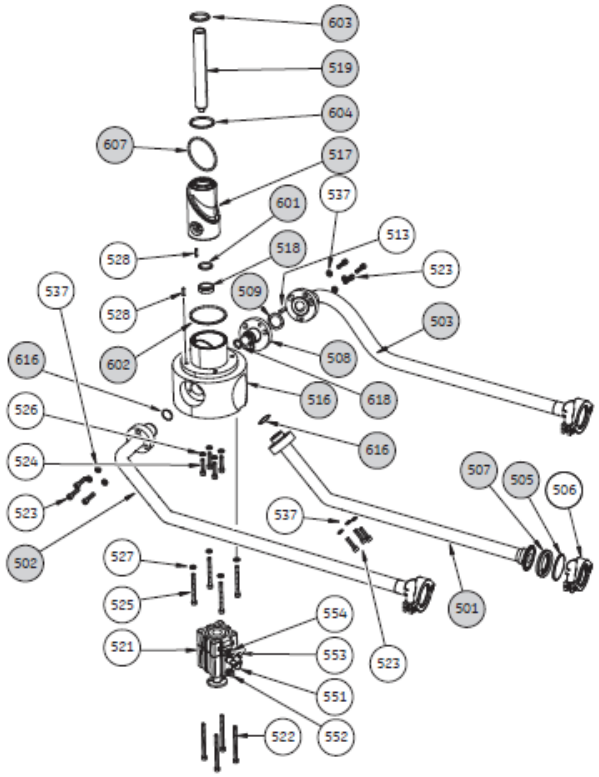


3.1.1.27.A.5 Media valve and tubes

Columns with stainless steel tubes and 800 and 1000 columns with PP tubes



300-600 columns with PP tubes



3.1.1.28.Profibus

11.4.5 AKTA Distributor: Regional Contact Information

Lebanon

Biopharm S.A.R.L.
P.O. Box 166418
Badaro-Sami el Solh Street
Beirut, Lebanon

Customer Service

Telephone 1: + 961 1 381 078

Fax nr: + 961 1 399 573

Email: mct@biopharmlb.com

Technical Support

Support to Algeria, Bahrain, Cyprus, Egypt, Greece, Iran, Iraq, Jordan, Kuwait, Lebanon, Malta, Morocco, Oman, Pakistan, Qatar, Republic of South Africa, Saudi Arabia, Syria, Tunisia, United Arab Emirates and Yemen.

Telephone 1: + 30 210 96 00 687

Fax nr: + 30 210 96 00 693

Email: service@hvd.gr

Instrument Service

Email: service@hvd.gr

11.5 Bioprocess modelled by software "SuperPro"

http://www.intelligen.com/superpro_overview.html

There can be downloaded a evaluation version. With this version diagrams can be built, but not stored (if they have more than 2 elements).

On the next sheet there is a process model example.

11.6 Recovery of recombinant S.cerevisae cells

From The Elements of Immunology , https://books.google.com.lb/books?isbn=8131711587, Fahim Halim Khan:

use is hepatitis B vaccine. A single gene for the major surface antigen of *hepatitis B virus* (HbsAg) is cloned in yeast cells. The recombinant yeast cells are grown in fermenters. HbsAg, the surface antigen, is expressed and accumulates inside the yeast cells. The yeast cells are then harvested and then burst open by high pressure releasing the recombinant HbsAg (among other proteins). HbsAg is then purified by a standard biochemical technique such as affinity chromatography. The purified antigen has been shown to induce humoral immunity. This approach has been used to make several

From Catherine Charcosset, Membrane Processes in Biotechnology and Pharmaceutics:

3.3.2 Concentration and clarification of cells

Another common application of MF is the concentration and washing of cultures of single-cell organisms where the product is intracellular or cell associated [7]. Common cases include recombinant yeast cultures producing proteins and antigen particles, and recombinant *E. coli* producing proteins in the form of solid inclusion bodies. The next step is usually cell lysis and recovery of the product from cell debris. Separation of product from cell debris can often be performed by MF, as detailed in the next section.

Russotti et al. [107] have used cross-flow MF for the harvest of recombinant yeast in a short period of time to minimize the risk for product degradation. MF studies with flat sheet membranes showed high throughput with initial fluxes on the order of water fluxes (>1000 l m^{-2} h^{-1}, regime I, <2 min), followed by a rapid decay towards a low pseudo-steady state flux (20 l m^{-2} h^{-1}, regime II, >2 min). Large pore membranes (0.65 μm) were found to be more suitable for harvesting yeast (10 μm size) without cell leakage than smaller pore ones (0.22 μm and 0.45 μm). Among operating parameters, feed flow rate (i.e. shear rate) had a significant impact on average flux, whereas change in *TMP* afforded little improvement. In another recent example, Lee [108] concentrated recombinant yeast cells using a cross-flow MF unit containing a 0.2 μm membrane device. The concentrated cells were homogenized by several passes through a high-pressure homogenizer. The homogenate was then clarified using cross-flow MF. The clarified material was concentrated by UF and buffer-exchanged before delivering the material to down-stream for further purification.

3.3.3 Separation of products from fermentation broth

Microorganisms are sources of valuable enzymes, proteins and other bio-products. They produce two basic types of biological molecules: extracellular, which are excreted into a growth medium, and intracellular, which are retained inside the cytoplasm of the cells [9]. A variety of host microorganisms have been studied. The most often used organisms are *E. coli*, *S. cerevisiae* and *Bacillus subtilis*. Several other microbial strains have been used for production of microbial enzymes, such as *Aspergillus niger* and *Kluyveromyces fragilis* (for production of catalase), *Saccharomyces lactis* and *Kluyveromyces lactis* (β-galactosidase), *Bacillus coagulans* and

From Process Scale Bioseparations for the Biopharmaceutical Industry, edited by Abhinav A. Shukla, Mark R. Etzel, Shishir Gadam:

fouling due to media components, and influence of osmotic pressure. Patel et al. [21] have compared the different filter formats: pleated-sheet microfilter, tubular microfilter, and hollow fiber ultrafiltration (UF), in terms of flux and cell yields obtained with CFF of yeast cell suspensions. They found that the UF module had much lower fouling rate than with the pleated-sheet microfilter that had rapid plugging and significant cleaning issues. Bailey and Meagher [27] performed a similar comparison between the hollow fiber and plate and frame formats for microfiltration of recombinant *Escherichia coli* lysate and found both options to be comparable in performance under optimized conditions. Sheehan et al. [22] performed a comparison of the centrifugation vs. membrane-based separations of extracellular bacterial protease and found the membrane process to be twice as cost effective as the centrifuge and equivalent to a precoated filter, on the basis of unit cost of enzyme product recovered. Industrial studies demonstrating robust operation of tangential flow filtration (TFF) for harvest of mammalian cell culture [23] and CFF for harvest of recombinant yeast cell product [26] have also been reported. More fundamental studies investigating the various aspects of filtration processes such as membrane fouling, mathematical modeling, and critical flux determination have also been published [24,28,29].

Pilot-scale harvest of recombinant yeast employing microfiltration: a case study, Gregory Russotti[a], A.Edward Osawa[a], Robert D. Sitrin[a], Barry C. Buckland[a], William R. Adams[b], Steven S. Lee[a]

Abstract

In order to develop a cost-effective recovery process for an intracellular product, crossflow microfiltration was studied for the harvest of a recombinant yeast under severe time constraint. It was required to process yeast broth in a short period of time to minimize the risk for product degradation. Preliminary microfiltration studies employing flat sheet membranes showed high throughout with initial fluxes on the order of water fluxes (> 1000 LMH, regime I, $2 min), followed by a rapid decay towards a low pseudo-steady state flux (20 LMH, regime II, > 2 min). Exploitation of these high fluxes and control of their eventual decline were crucial in establishing a rapid crossflow filtration process. The effect of several parameters, such as initial cell concentration, shear rate, transmembrane pressure, membrane pore size and medium composition on filtration performance were investigated to better understand the flux decline mechanisms. We found that the major contributor to flux decay was reversible fouling by the cake formation on the membrane surface. Within the operating boundaries of our microfiltration system, large-pore membrane (0.65 μm) was much more desirable for harvesting our yeast (10 μ size) without cell leakage than smaller pore ones (0.22 μm and 0.45 μm). Among adjustable operating parameters, feed flow rate (i.e., shear rate) exerted significant impact on average flux, whereas manipulation of transmembrane pressure afforded little improvement. Although initial cell concentration affected adversely the permeation rates, growth medium components, especially soy-peptone, was deemed pivotal in determining the characteristics of cell cake, thus controlling yeast microfiltration.

Keywords: Crossflow filtration; Microfiltration; *Saccharomyces cerevisiae*; Yeast; Recombinant

Biotechnology Techniques Vol 4 No 5 329-334 (1990)
Received 12th June

DISRUPTION OF *Saccharomyces cerevisiae* USING ENZYMATIC LYSIS COMBINED WITH HIGH-PRESSURE HOMOGENIZATION

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Department of Chemical Engineering, University of Waterloo,
Waterloo, Ontario, Canada N2L 3G1

ABSTRACT

The disruption of commercially-available pressed Bakers' yeast (*Saccharomyces cerevisiae*) was studied using a relatively new high-pressure homogenizer (the Microfluidizer). Initial experiments using only mechanical disruption generally gave low disruption yields (i.e., less than 40 % disruption in 5 passes). Consequently combinations of two disruption methods, namely enzymatic lysis and subsequent homogenization, were tested to identify achievable levels of disruption. The enzyme preparation employed was Zymolyase, which has been shown to effectively lyse the walls of viable yeast. Yeast cell suspensions ranging in concentration from 0.6 to 15 gDW/L were disrupted with and without enzymatic pre-treatment. Final total disruption obtained using the combined protocol approached 100 % with 4 passes at a pressure of 95 MPa, as compared to only 32 % disruption with 4 passes at 95 MPa using only homogenization. A model is presented to predict the fraction disrupted while employing this novel enzymatic pre-treatment.

INTRODUCTION

An abundance of valuable biochemicals can be produced by microorganisms. Some of these products can be made to be secreted by the microorganism; however in some cases this is not possible to achieve and the cells must be disrupted to release their contents. There are many different methods for cell disintegration. Some of these methods can be used in association with one another, in order to take advantage of their combined benefits [1].

The Microfluidizer, a high-pressure homogenizer, has recently been tested with native and recombinant strains of *E. coli* [2]. The cell suspension is forced through two parallel channels (2x100 µm) and the separate high-velocity streams are directed at each other in front of a wall.

Preliminary disruption experiments with *Saccharomyces cerevisiae* using Microfluidization alone indicated the need for an improved disruption methodology. A combination of methods was suggested and an enzymatic pre-treatment using Zymolyase to "soften up" the cells was employed.

Zymolyase-20T is commercially available from Seikagaku Kogyo Co., Ltd. It is produced by a submerged culture of *Oerskovia xanthineolytica* (previously classified as *Arthrobacter luteus*). An essential enzyme responsible for lysis of viable yeast cells in this preparation is β-1,3-glucan laminaripentaohydrolase [3-6].

Using Zymolyase as a pre-treatment to Microfluidization, a study of various homogenization operating conditions (i.e., disruption pressure and number of passes) has been undertaken in order to determine the acceptability of using the Microfluidizer as a method for mechanical disruption of yeast. An empirical model is presented to account for the introduction of this novel enzymatic pre-treatment regime.

MATERIALS AND METHODS

ENZYME EMPLOYED FOR PRE-TREATMENT

Zymolyase-20T, produced by a submerged culture of *Oerskovia xanthineolytica*, is a relatively new enzyme preparation which effectively lyses the walls of viable yeast cells [7,8]. Zymolyase-20T has 20,000 units/g of lytic activity, defined below, toward Brewers' yeast (*Saccharomyces uvarum*, resting stage).

One unit of lytic activity is defined as that amount which results in a 30 % decrease in absorbance at 800 nm (A 800) of the reaction mixture under the following conditions:

Reaction Mixture	
Enzyme.....0.1 mg/mL solution	1 mL
Substrate.....Yeast Cell Suspension (2 mgDW/mL)	3 mL
Buffer.....M/15 Phosphate Buffer pH 7.5	5 mL
Deionized water	1 mL
Total volume	10 mL

After incubation for 2 h at 25 °C with gentle shaking, A 800 of the mixture is determined. As a reference, 1 mL of deionized water is used instead of the enzyme solution.

$$\% \text{ decrease in A 800} = \frac{(\text{A 800 ref.} - \text{A 800 react. mixture})}{\text{initial A 800 ref.}} \times 100\%$$

When an A 800 decrease of 60 %, equivalent to 2 units of activity, is observed in the reaction system, the yeast cells are completely lysed, namely 1 unit of Zymolyase-20T lyses 3 mg dry weight of Brewers' yeast [9].

DISRUPTION EQUIPMENT

The disruption device used in this work was a Microfluidizer high-pressure homogenizer (model M110T with extra heavy duty pump; Microfluidics Corp., Newton, MA., U.S.). The disruptor consisted of an air-driven, high-pressure pump (ratio 1:250; required air pressure 0.6-1 MPa) and a special disruption chamber with an additional back pressure unit. A minimum sample size of 20 mL is required for processing. Further details are given in [2].

STRAINS AND ANALYTICAL

Commercially-available pressed Bakers' yeast (*Saccharomyces cerevisiae*) was resuspended in deionized water to a cell concentration of 2 mgDW/mL. The resulting reaction mixture thus has a yeast cell concentration of 0.6 gDW/L. The above enzymatic pre-treatment (prior to homogenization) was carried out for 2 h, with the percent decrease in A 800 found at the end of the 2 h incubation (the course of the enzymatic lysis was followed by sampling the reaction and reference mixtures every 15 min.).

The buffered and partially lysed yeast suspension after enzymatic pre-treatment was cooled to 4 °C and was subsequently homogenized at various operating pressures (30-95 MPa) and at up to five passes through the homogenizer. The resulting homogenized yeast suspensions were cooled using ice packs, as was the disruption chamber. The Microfluidizer

11.6.1 How to Use Avestin Emulsiflex C3 Homogenizer to Disrupt Cells

Cell Biology > Cell viability > Cell lysis

Fungi > Saccharomyces > Saccharomyces cerevisiae > Other compound

Author: Zongtian Tong

1/5/2011, 7257 views, 1 Q&A

[Abstract] The EmulsiFlex-C3 homogenizer is powered by an electric motor. The pump does not require a compressor for it to run. This equipment can be used to disrupt cells at a large scale. The EmulsiFlex-C3 has a fixed flow-through capacity of 3L/h. It has the ability to process samples as small as 10 ml. The homogenizing pressure is adjustable between 500 and 30,000 psi. In this protocol, we describe the use of the Avestin Emulsiflex C3 Homogenizer to disrupt *S. pombe* and *S. cerevisiae* cells.

Equipment

Figure 1. Avestin Emulsiflex C3 homogenizer

Procedure

1. Switch on the homogenizer at the back.
2. Turn on nitrogen. Pressure reads 80 psi.
3. Unscrew the funnel cap. Check if the funnel cap is on to make sure ethanol does not evaporate.
4. Turn red stop knob close wise and push green knob to start.
5. Pump residual ethanol out of the tubing.
6. Pour DI water into the funnel to wash ethanol out. Keep air pressure on occasionally to make sure no cell debris is left from the last user.
7. Before load your samples, take the funnel off and roll it on ice to keep it cool. Install the funnel back to the top. Put the steel coil heat exchanger into ice to cool down the samples.
8. Load your samples into the funnel. Turn on the homogenizer. Let the samples run through the tubing back to the funnel before air pressure is on.

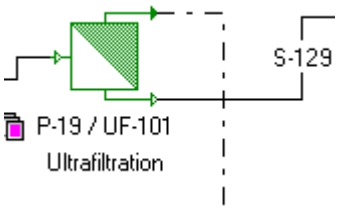
9. Turn on air pressure. Air pressure at 40 psi, gauge pressure ≥ 20,000 psi and < 25,000 psi. The maximum pressure is 30,000 psi. Leave the tubing in a sample collection tube chilled on ice.
10. *S. Pombe* samples need to be passed through 5~6 times to reach 80~90% efficiency. *S. cerevisiae* samples need to be passed through 8~9 times to reach 80~90% lysis efficiency. Check samples under a microscope.
11. If the homogenizer is clogged by the samples, cap the funnel and blow with nitrogen tube.
12. After samples are done, take off the funnel and rinse it with DI water.
13. Run more water to flush out cell debris. Keep the air pressure on occasionally.
14. Run ethanol and leave 1/3 of a funnel volume of ethanol in the funnel.

References

1. http://www.avestin.com/English/c3page.html

How to cite this protocol: (2011). How to Use Avestin Emulsiflex C3 Homogenizer to Disrupt Cells. *Bio-protocol* Bio101: e11. http://www.bio-protocol.org/e11

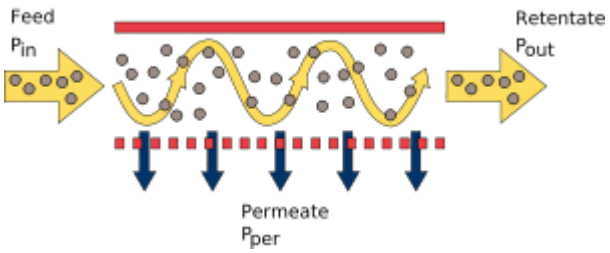
11.7 Crossflow filtration



The whey proteins are concentrated using crossflow ultrafilters (UF-101).

From Wikipedia, the free encyclopedia

Jump to: navigation, search



In chemical engineering, biochemical engineering and protein purification, crossflow filtration[1] (also known as tangential flow filtration[2]) is a type of filtration (a particular unit operation). Crossflow filtration is different from dead-end filtration in which the feed is passed through a membrane or bed, the solids being trapped in the filter and the filtrate being released at

the other end. Cross-flow filtration gets its name because the majority of the feed flow travels tangentially across the surface of the filter, rather than into the filter.[1] The principal advantage of this is that the filter cake (which can blind the filter) is substantially washed away during the filtration process, increasing the length of time that a filter unit can be operational. It can be a continuous process, unlike batch-wise dead-end filtration.

Besonders dafür geeignet sind Hohlfasern (Kapillarmembran oder auch Hohlfäden genannt), deren Leistungsfähigkeit noch durch den sogenannten Pinch-Effekt verstärkt wird. Eine übliche Hohlfaser hat einen Innendurchmesser von circa 1,5 mm (3,0 mm bis 0,1 µm möglich) und eine Porengröße von 200 bis 5 nm (2 µm bis 1,0 nm möglich). Je nach Anwendung werden hunderte bis tausende Kapillaren in Modulen zusammengefasst und vergossen (Hohlfasermodule). Mit Hilfe einer Zirkulationspumpe wird das unfiltrierte Produkt solange durch die Kapillaren zirkuliert, bis die Trubstoffe im Retentat so konzentriert sind, dass eine Entleerung und Reinigung erforderlich wird.

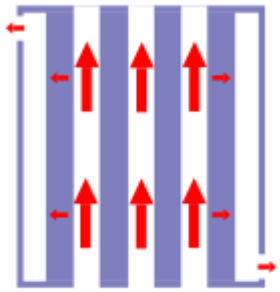


Diagram of cross-flow filtration

This type of filtration is typically selected for feeds containing a high proportion of small particle size solids (where the permeate is of most value) because solid material can quickly block (blind) the filter surface with dead-end filtration. Industrial examples of this include the extraction of soluble antibiotics from fermentation liquors.

Filtration unit for industrial cross-flow filtration

Charakteristische Merkmale der Cross-Flow-Filtrationstechnologie sind die weitgehende Eliminierung von Filterhilfsmitteln, d. h. deren Beschaffung, Lagerung, Handhabung und Entsorgung, die rasche, arbeitsextensive sowie die qualitätsschonende Verarbeitung.

Die Cross-Flow-Filtration ist sehr energieaufwändig. Ein großer Teil der in die Förderung des Feeds investierten Energie geht durch das Retentat verloren. Deshalb wird an den Stellen, wo darauf verzichtet werden kann, immer stärker auf die Dead-End-Filtration zurückgegriffen.

11.8 Dead-End Filtration

To be described

11.9 Anion exchange chromatography[45]

Anion-exchange chromatography is a process that separates substances based on their charges using an ion-exchange resin containing positively charged groups, such as diethyl-aminoethyl groups (DEAE).[2] In solution, the resin is coated with positively charged counter-ions (cations). Anion exchange resins will bind to negatively charged molecules, displacing the counter-ion. Anion exchange chromatography is commonly used to purify proteins, amino acids, sugars/carbohydrates and other acidic substances [3] with a negative charge at higher pH levels. The tightness of the binding between the substance and the resin is based on the strength of the negative charge of the substance.

General technique for protein purification

A slurry of resin, such as DEAE-Sephadex is poured into the column. After settling, the column is pre-equilibrated in buffer before the protein mixture is applied. Unbound proteins are collected in

[45] http://en.wikipedia.org/wiki/Anion-exchange_chromatography

the flow-through and/or in subsequent buffer washes. Proteins that bind to the resin are retained and can be eluted one of two ways. First, the salt concentration in the elution buffer is gradually increased. The negative ions in the salt solution (e.g. Cl-) compete with protein in binding to the resin. Second, the pH of the solution can be gradually decreased which results in a more positive charge on the protein, releasing it from the resin. As buffer elutes from the column, the samples are collected using a fraction collector.

11.10 Final product formulation[46]

High-resolution chromatography normally yields a protein that is 98-99 per cent pure.

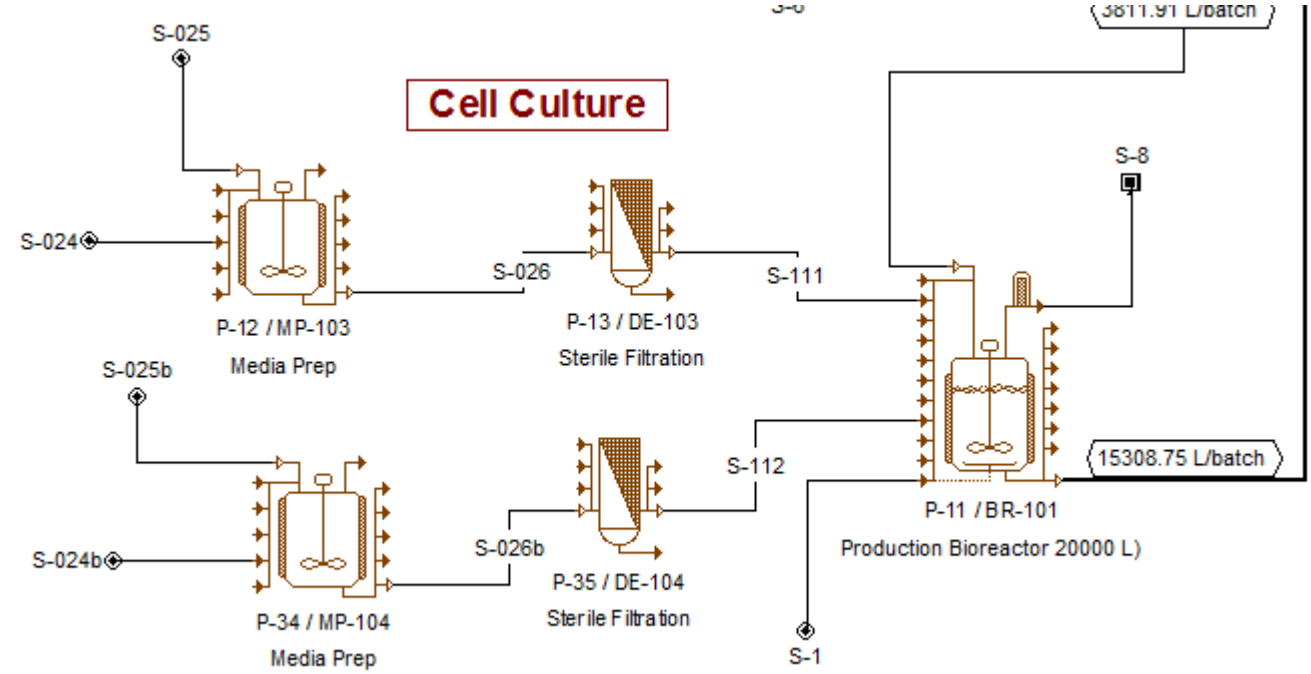
Final product formulation bings the product in the final format. This envolves:

- Addition of various excipients
- Filtration of the final product through a 0.22 μm absoulute filter, then aseptic filling into final containers.
- Freeze-drying if the product is to be marketed in a powedered format.

Fig.: 0.22 μm filter

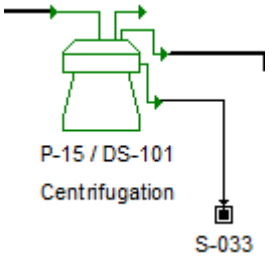
[46] [Walsh 2007], ch.6.9

11.11 Bioreactor/Tank

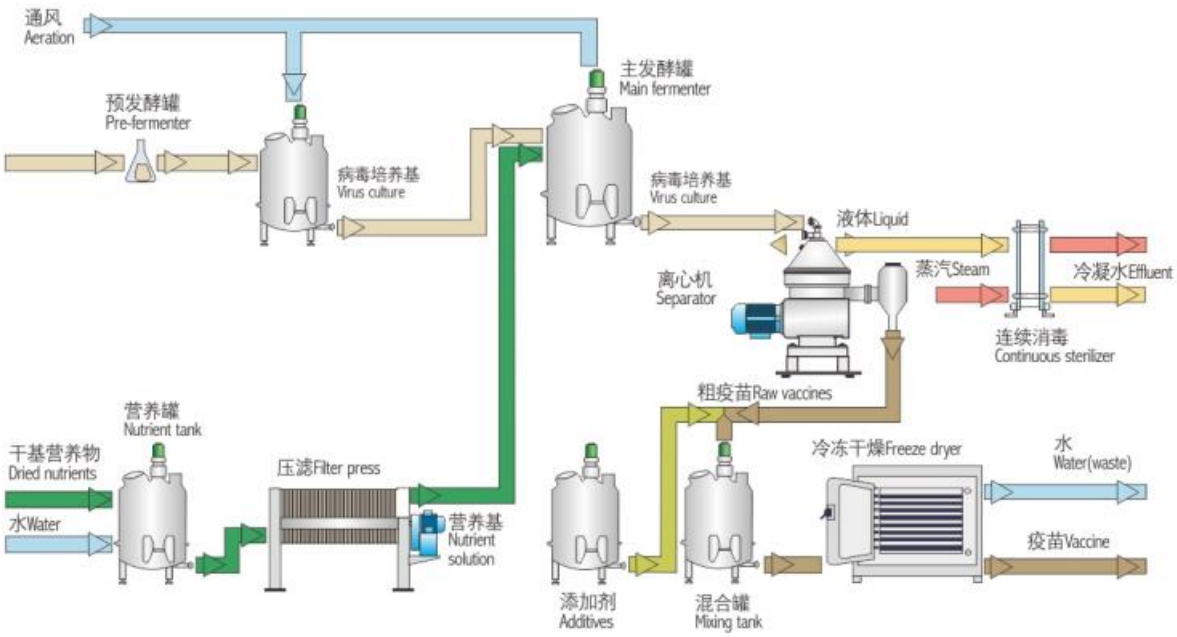


S.cerevisiae needs as nutrition only sugar.

11.12 Disc-Stack Centrifuge



11.12.1 Example for Supplier: Huanding China



型号 Model	处理量 Capacity(L/h)	重量 Weight(Kg)	外型尺寸(长*宽*高) Dimension(L*W*H)
BTSX15	400~1000	1100	950×950×1250
BTSX35	1000~3000	1500	1400×1400×1450
BTSX85	3000~70000	1800	1750×1450×1850
BTSX150	14000-30000	3700	1850×1500×1850
BTSX200	20000-40000	4800	2100×1700×2200

2014-12-26 13:57 GMT+02:00 Chouse Hu <chousehu@huading-separator.com>:

Dear Samir Mourad

do you think $250,000 dollar in your budget ?

Best Regards

Chouse Hu

On Dec 27, 2014 8:50 AM, "Chouse Hu" <chousehu@huading-separator.com> wrote:

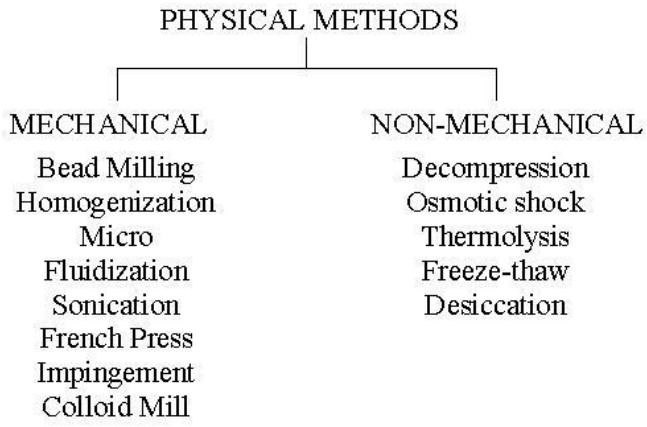
our cheapest centrifuge can be used in this case at all, that means nothing for you.

please refer to Westalia Separator, and learn what a particular centrifuge can meet your demand.

then, you could try to find out an alternative from China, to reduce your cost.

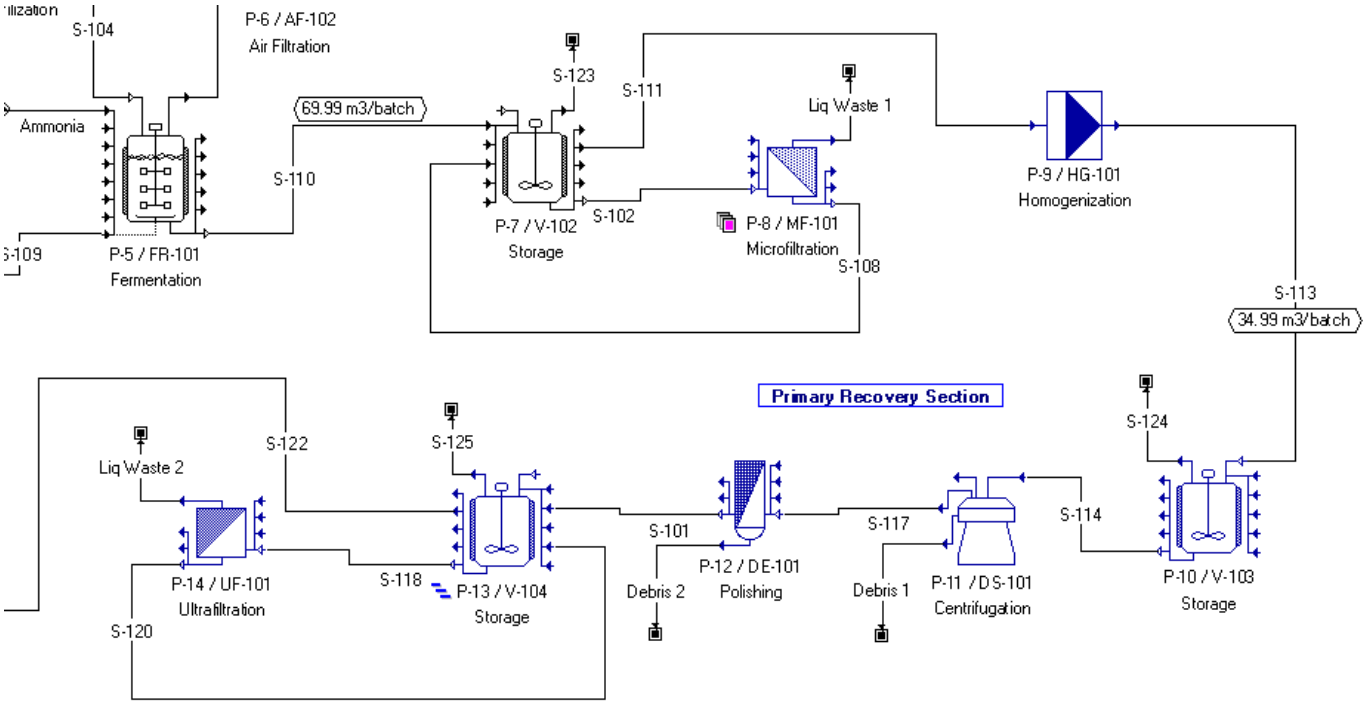
http://www.westfalia-separator.com/no_cache/contact/locations-worldwide/view.html

11.13 Homogenization



Method of cell lysis	disadvantages
freeze-thaw	very slow
chemical lysis	can cause changes in protein structure, difficulties in purification, expensive detergents
enzymatic lysis	often not reproducible, enzyme stability, long incubation time, necessity of removing the lysis enzymes, expensive scale-up, often combination with other method necessary
cell bomb	only applicable to specific cell types
high pressure homogenizer (e.g. french press)	expensive equipment, high maintenance due prone of orifices' clogging
centrifugation	only for very weak cell walls
ball mill / bead mill	uneven processing = incomplete lysis, protein denaturation, low efficiency whilst relatively high energy consumption, complex separation of milling medium and product, time-consuming cleaning

11.13.1 Principle



11.13.2 Suppliers

11.13.2.1 Ningbo Scientz company

High Pressure Homogenizer JG-IA Series

Acting on high-pressure principle, the instrument is used for extruding cells, especially suitable for smashing thick-wall cells, germs and denser solution samples. Having no noise, less temperature rise and no metal ion contamination, it has wide application of such research fields as protein study, nucleic acid extraction, cell disruption in genetic engineering labs of colleges, scientific research institutes and pharmaceutical factories.

● Specifications:

Model	JG－IA	Capacity	50ml/times Continuous increase samples for scientific research
Voltage	380 VAC	Maximum operating pressure	256 Mpa (37120 psi)
Pressure device	Hydraulic System	Max Pressure stroke	170 mm
Sample tube	Φ25mm Stainless	Pressure plate speed	6.8 mm/s
Dimensions	555×600×1170 mm	Largest volume of sample	50 ml/times

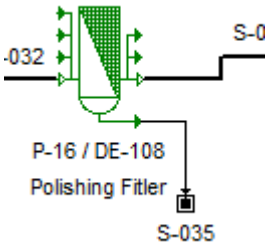
11.13.2.2 Zhangjiagang Beyond Machinery Co., Ltd.

Model Number: GJB, Shipping Terms: FOB Port: Shanghai

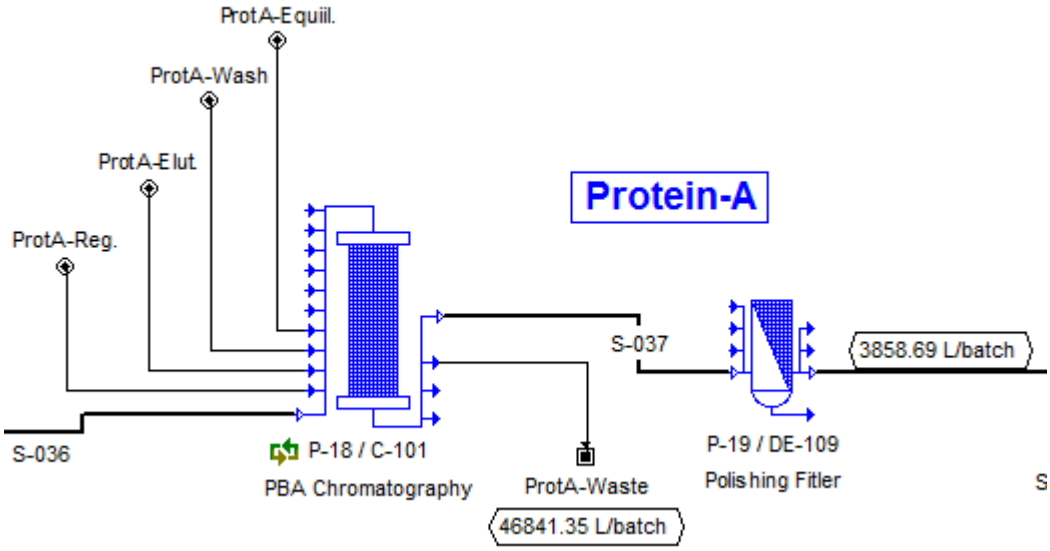
Unit Price: USD 10,000/Sets quantity: 1 Sets

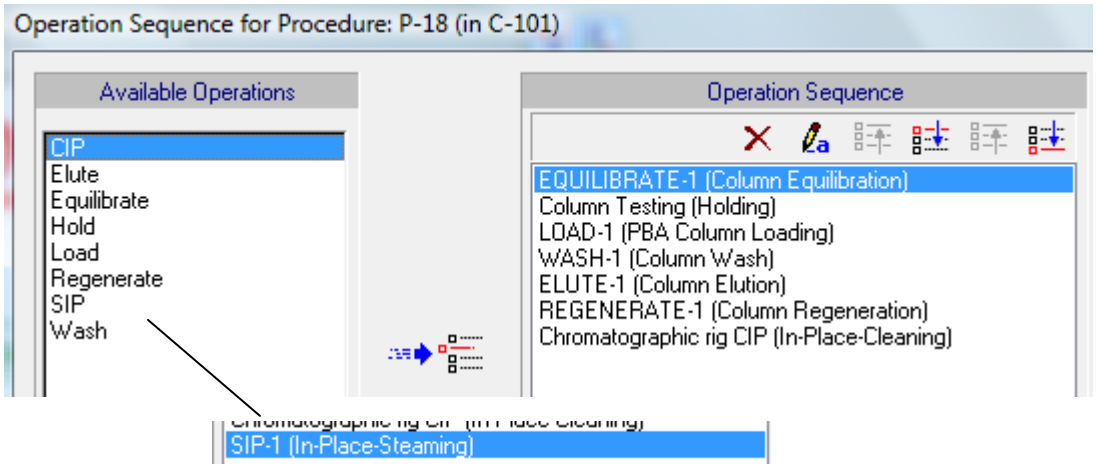
Lead Time: 30days Payment Terms: T/T Quotation Valid Till: 2015-1-28

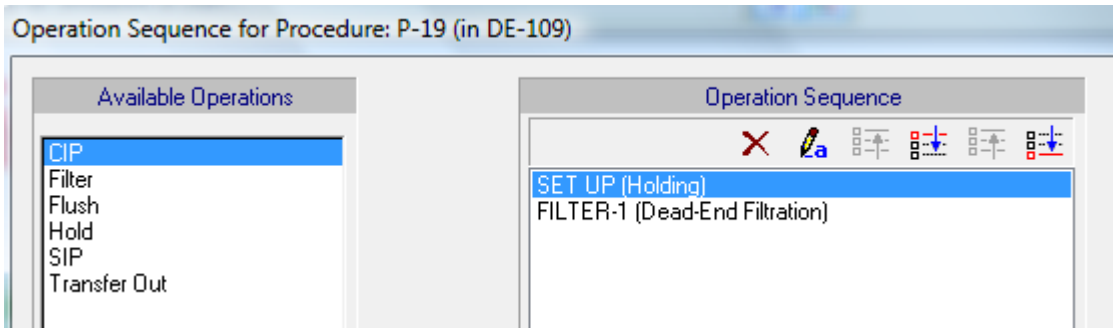
11.14 Dead-End Filter



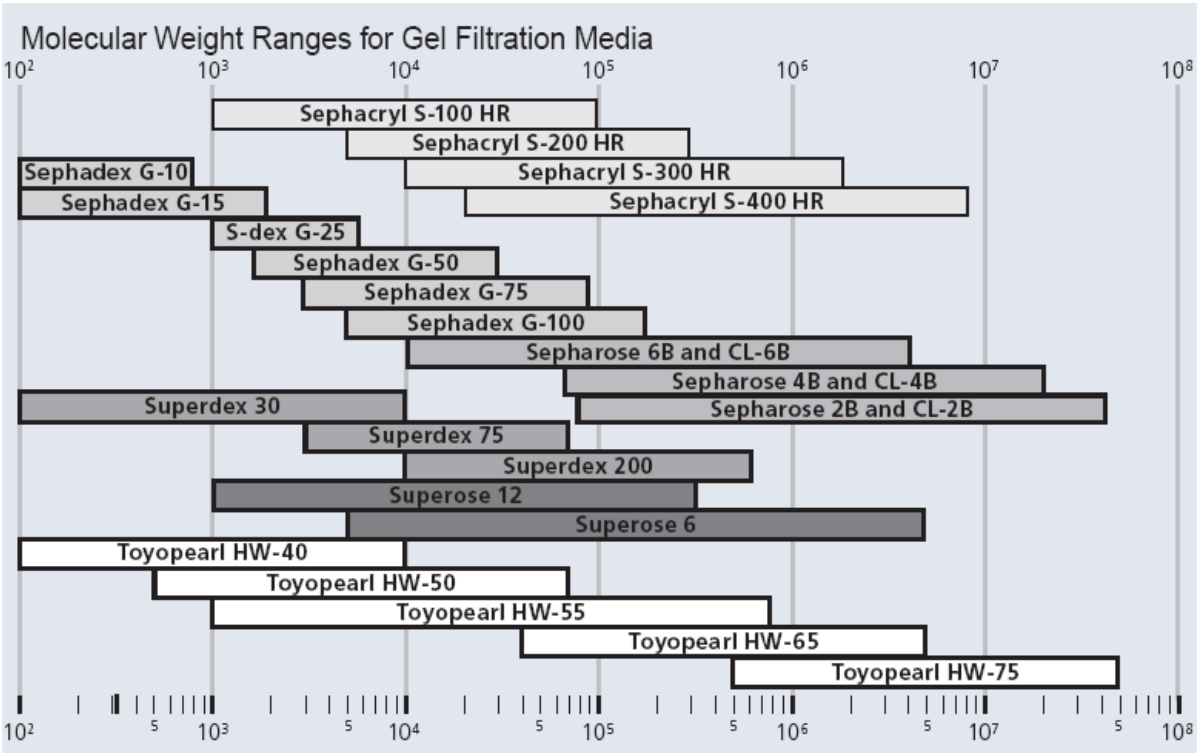
11.15 Protein-A Chromatography



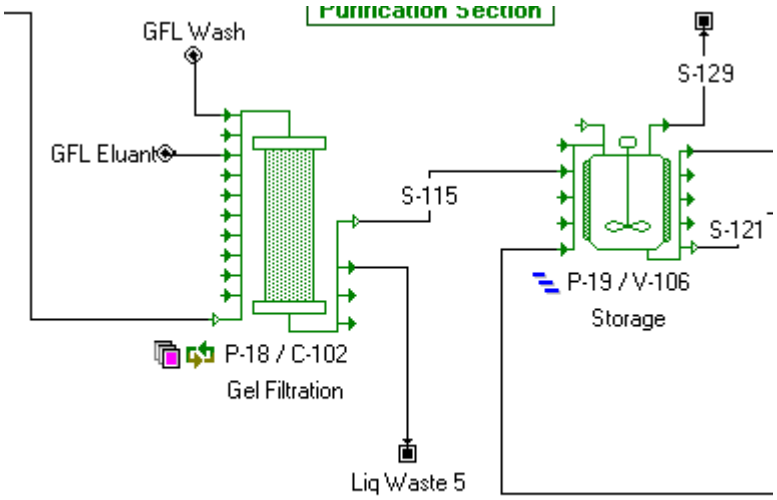




11.16 Gel filtration chromatography



4B Sepharose



11.16.1 sensor/actuator list

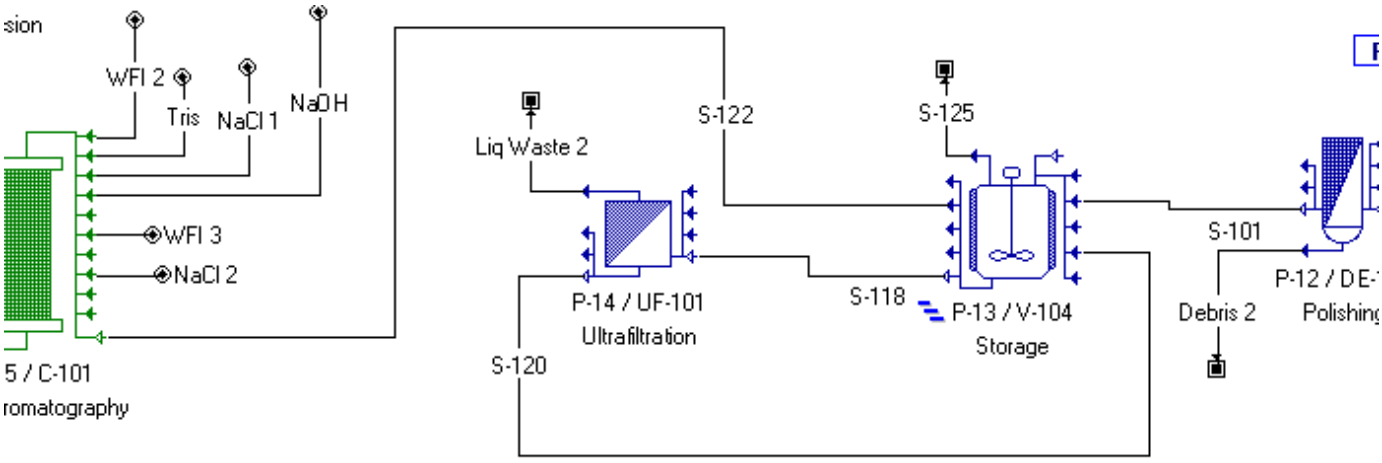
similiar to AKTA process

Akta process Sensors and actuators 13.12.13

Teil	**Anzahl**	Item Price
Air trap	1	
Filter	1	
Filter vent valve	1	
Capsule filter bottom manual valve	1	
Capsule filter top manual valve	1	
System pump	2	
Sample pump	1	
Pressure control valve	2	
Buffer A inlet valves	10	
Buffer B inlet valves	6	
Sample connection valve	1	
Sample inlets valves	2	
Air trap inlet valve	1	
Air trap bypass valve	1	
Air trap vent valve	1	
Air trap outlet valve	1	
Filter inlet valve	1	
Filter bypass valve	1	
Filter outlet valve	1	
System connection valve	1	
Column 1 top inlet valve	1	
Column 1 bottom inlet valve	1	
Column 1 top valve	1	
Column 1 bottom valve	1	
Column 1 top outlet valve	1	
Column 1 bottom outlet valve	1	

Column 2 top inlet valve	1
Column 2 bottom inlet valve	1
Column 2 top valve	1
Column 2 bottom valve	1
Column 2 top outlet valve	1
Column 2 bottom outlet valve	1
Outlet valves	9
Air trap drain valve	1
Filter drain valve	1
CIP / AxiChrom manifold	1
Buffer inlet air sensor	1
Pre-column air sensor	1
Post-column pH-meter	1
Post-column UV-meter	1
Pre-column conductivity meter	1
Post-column conductivity	1
System flow meter	1
Air trap high level meter	1
Air trap low level meter	1
Pre-filter pressure meter	1
Pre-column pressure meter	1
Sample pump pressure meter	1
PCV pressure meter, A inlets	1
PCV pressure meter, B inlets	1

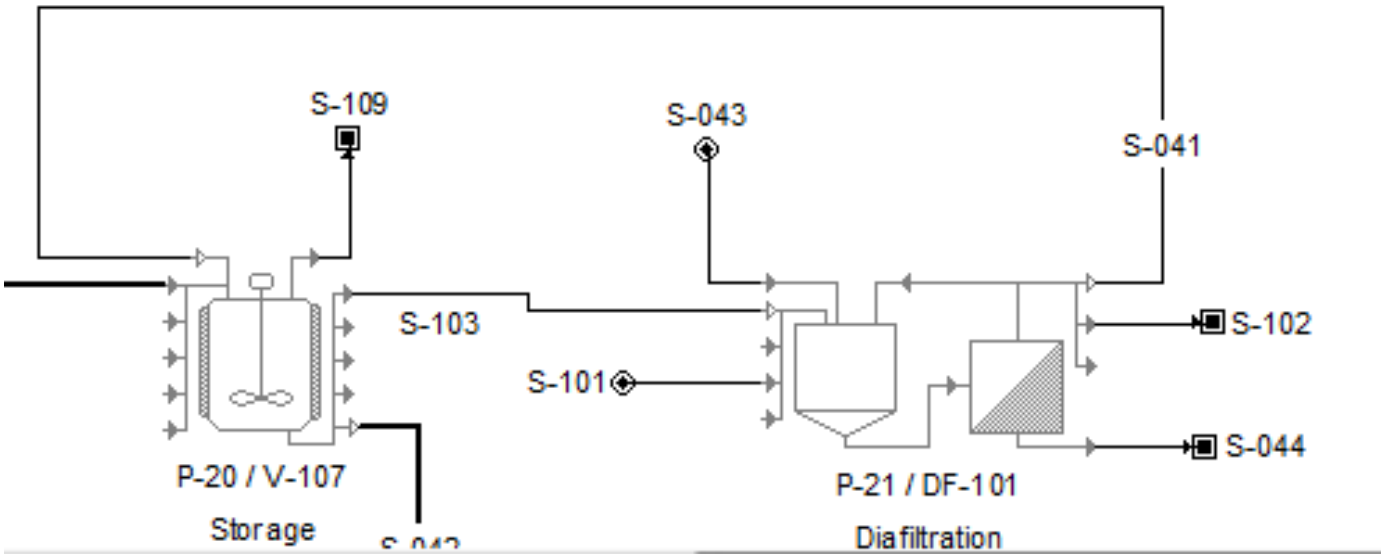
11.17 Ultrafiltration

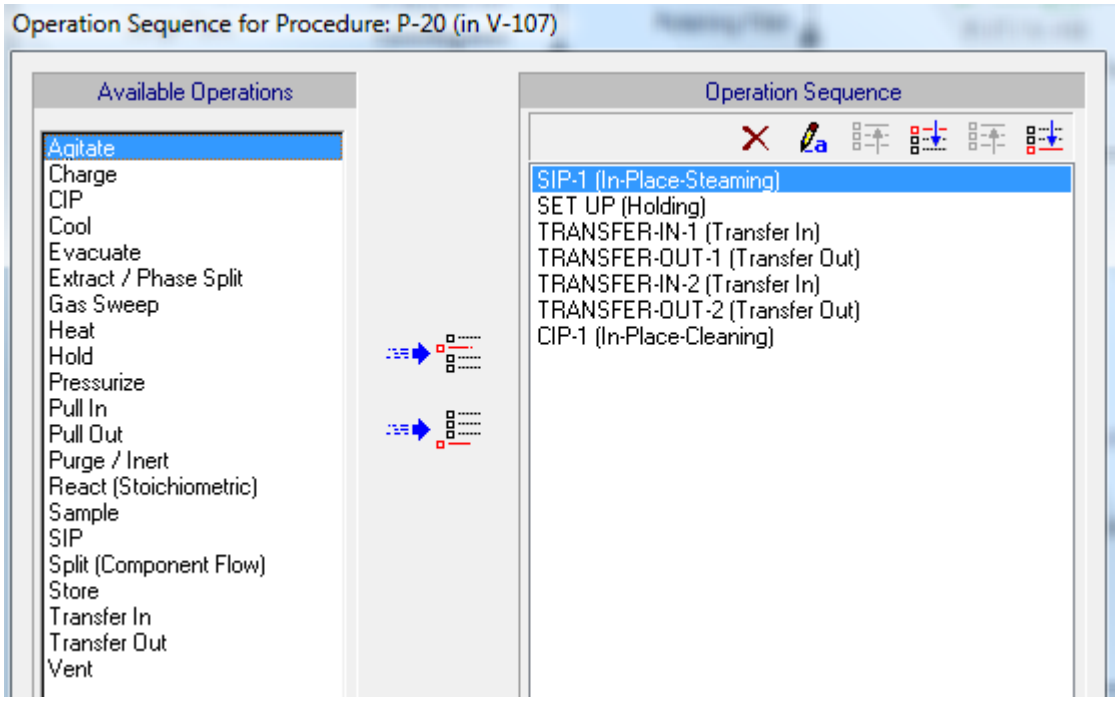


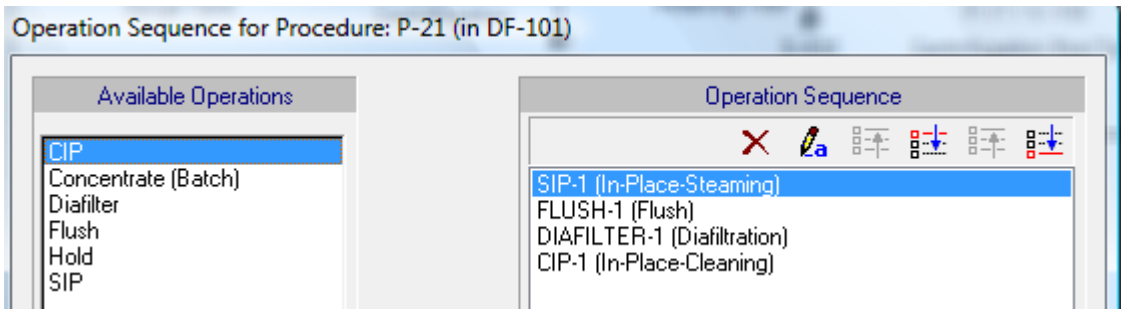
From SuperPro Example Bgal

11.18 Diafiltration

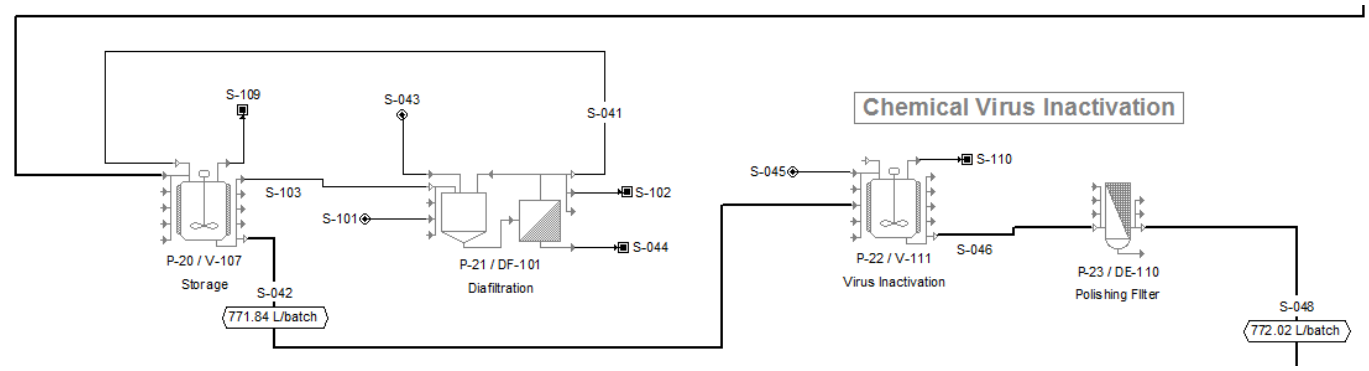
Same equipment as ultrafiltration

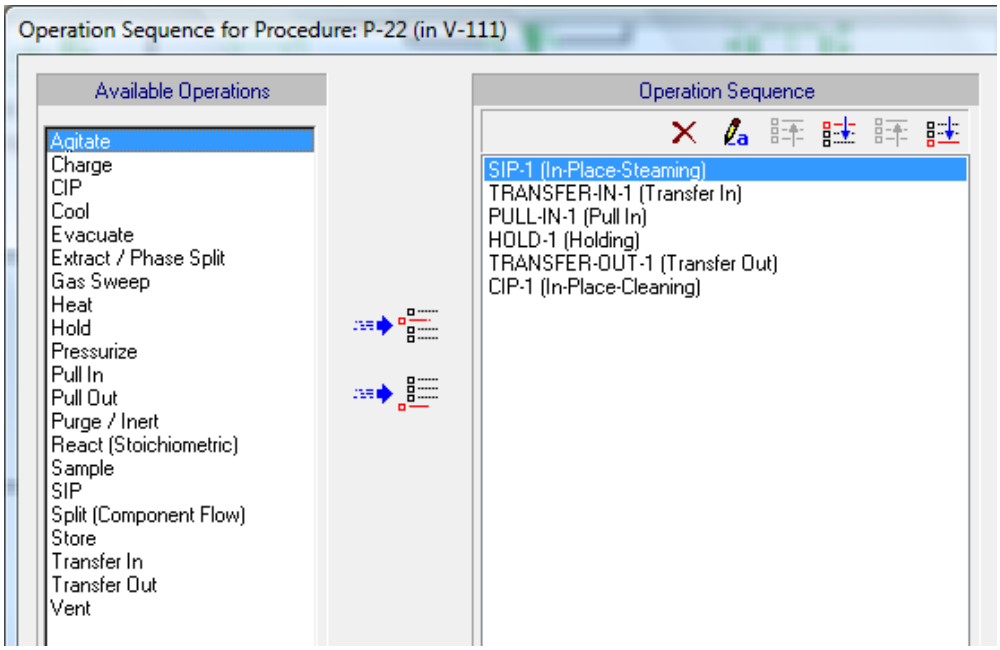






11.19 Chemical Virus Inactivation

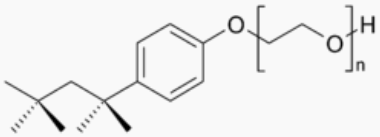




11.19.1 Solvent/detergent (S/D) inactivation[47]

This process, developed by the New York Blood Center,[5] is the most widely used viral inactivation method to date. It is predominantly used in the blood plasma industry, by over 50 organizations worldwide and by the American Red Cross [1]. This process is only effective for viruses enveloped in a lipid coat, however. The detergents used in this method interrupt the interactions between the molecules in the virus's lipid coating. Most enveloped viruses cannot exist without their lipid coating so are destroyed when exposed to these detergents. Other viruses may not be destroyed but they are unable to reproduce rendering them non-infective. The solvent creates an environment in which the aggregation reaction between the lipid coat and the detergent happen more rapidly. The detergent typically used is Triton-X 100.

[47] http://en.wikipedia.org/wiki/Virus_processing#Viral_inactivation



Chemical Structure of Triton X-100 (n = 9-10).

This process has many of the advantages of the "traditional" removal techniques. This process does not denature proteins, because the detergents only affect lipids and lipid derivatives. There is a 100% viral death achieved by this process and the equipment is relatively simple and easy to use. Equipment designed to purify post-virus inactivated material would be necessary to guard against contamination of subsequent process streams.

S/D treatment utilizes readily available and relatively inexpensive reagents, but these reagents must be removed from the product prior to distribution which would require extra process steps. Because this process removes/inactivates the lipid coating of a virus, viruses without any sort of lipid envelope will be unaffected. There is also no inactivation effect by the buffers used in this process.

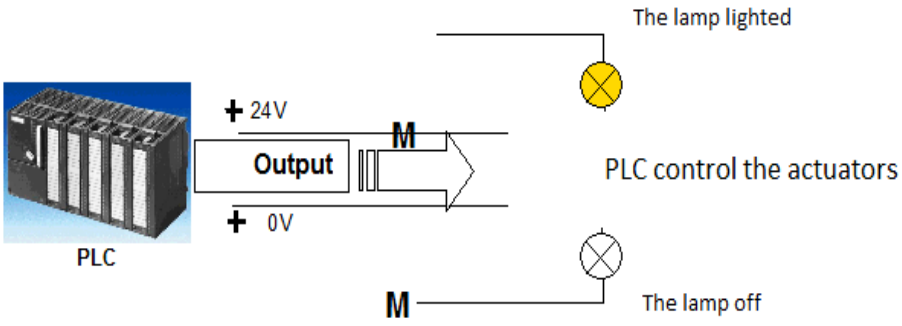
11.20 Introduction to Siemens S7 PLC system

11.20.1 Definition of PLC:

PLC is an acronym for Programmable Logic Control (logic controllers programmable). This description of the system that controls the process (exp machine to print newspapers, facility for packing cement, piston to cut plastic ...). This process is carried out in accordance with the instructions of the program in the device memory.

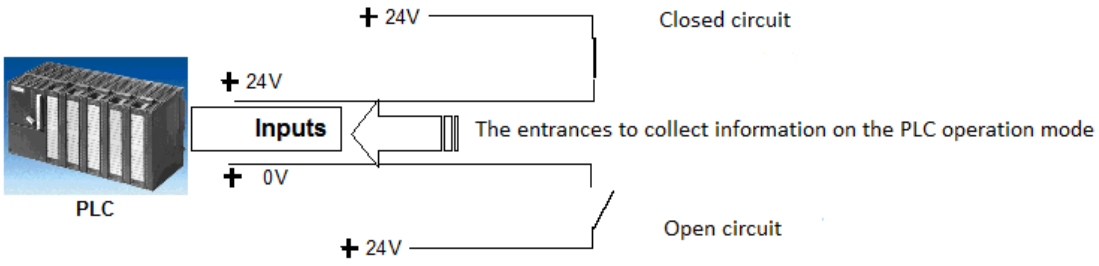
11.20.2 Function of PLC:

PLC controls the process in which the actuators are connecting to links feed (for example, 24 volts) specific to the PLC exits Outputs. Through these links we can run and turn off the engine, open and close the valves, or turn on and turn off the lights.

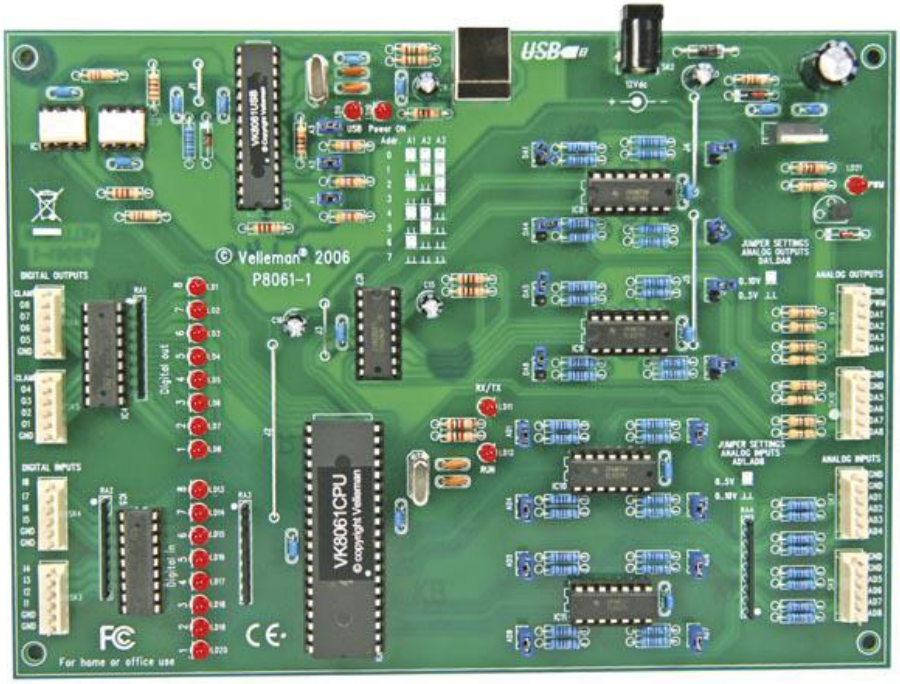


11.20.3 Where to get the information about the PLC operation mode:

PLC receives the information about the operation from the signal - generator connected to the entrances of the PLC. These signal generators can be, for example, sensors recognize the status of the working parts, keys or buttons. This specific situation can be open or closed. The difference between Note normally open NC: Normally Closed is ineffective when they are closed and petitions NO: Normally Open Normally open that are not effective when they are open:



11.21 Introduction to the DLL for the USB Interface Board K8061:

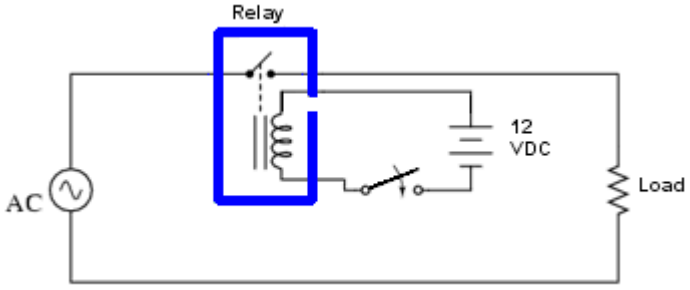


The K8061 interface board has 8 digital input channels and 8 digital output channels. In addition, there are 8 analogue inputs, 8 analogue outputs and one PWM output. The number of inputs/outputs can be further expanded by connecting more (up to a maximum of eight) cards to the PC's USB connectors. Each card is given its own identification number by means of three jumpers, A1, A2 and A3 (see table 1 below for card numbering).

Connection to the computer is optically isolated, so that damage to the computer from the card is not possible.

11.22 Definition of a relay

A relay is usually an electromechanical device that is actuated by an electrical current. The current flowing in one circuit causes the opening or closing of another circuit. Relays are like remote control switcher and are used in many applications because of their relative simplicity, long life, and proven high reliability. Relays are used in a wide variety of applications throughout industry, such as in telephone exchanges, digital



computers and automation systems. Highly sophisticated relays are utilized to protect electric power systems against trouble and power blackouts as well as to regulate and control the generation and distribution of power.

How do relays work?

All relays contain a sensing unit, the electric coil, which is powered by AC or DC current. When the applied current or voltage exceeds a threshold value, the coil activates the armature, which operates either to close the open contacts or to open the closed contacts. When a power is supplied to the coil, it generates a magnetic force that actuates the switch mechanism. The magnetic force is, in effect, relaying the action from one circuit to another. The first circuit is called the control circuit; the second is called the load circuit.
There are three basic functions of a relay: On/Off Control, Limit Control and Logic Operation.

Specification, System Design, Detailed Devices and Plant Design, and Automation for the MEGBI Vaccine Pilot Plant MEGBI-VPP[48]

[48] From [MEGBI-VPP 2012] and [MEGBI-VPP 2013]

12 Chromatographic Purification Device: Specification

12.1 sensor/actuator list

similiar to AKTA process

Akta process Sensors and actuators 13.12.13		
Teil	**Anzahl**	Item Price
Air trap	1	
Filter	1	
Filter vent valve	1	
Capsule filter bottom manual valve	1	
Capsule filter top manual valve	1	
System pump	2	
Sample pump	1	
Pressure control valve	2	
Buffer A inlet valves	10	
Buffer B inlet valves	6	
Sample connection valve	1	
Sample inlets valves	2	
Air trap inlet valve	1	
Air trap bypass valve	1	
Air trap vent valve	1	
Air trap outlet valve	1	
Filter inlet valve	1	
Filter bypass valve	1	
Filter outlet valve	1	
System connection valve	1	
Column 1 top inlet valve	1	
Column 1 bottom inlet valve	1	
Column 1 top valve	1	
Column 1 bottom valve	1	
Column 1 top outlet valve	1	
Column 1 bottom outlet valve	1	
Column 2 top inlet valve	1	
Column 2 bottom inlet valve	1	
Column 2 top valve	1	
Column 2 bottom valve	1	
Column 2 top outlet valve	1	
Column 2 bottom outlet valve	1	
Outlet valves	9	
Air trap drain valve	1	
Filter drain valve	1	
CIP / AxiChrom manifold	1	

Buffer inlet air sensor	1
Pre-column air sensor	1
Post-column pH-meter	1
Post-column UV-meter	1
Pre-column conductivity meter	1
Post-column conductivity	1
System flow meter	1
Air trap high level meter	1
Air trap low level meter	1
Pre-filter pressure meter	1
Pre-column pressure meter	1
Sample pump pressure meter	1
PCV pressure meter, A inlets	1
PCV pressure meter, B inlets	1

13 Concept

13.1 Mechanical structure

The concept is to install a simplified DNA vaccine production line based on devices of GE Health and other suppliers.

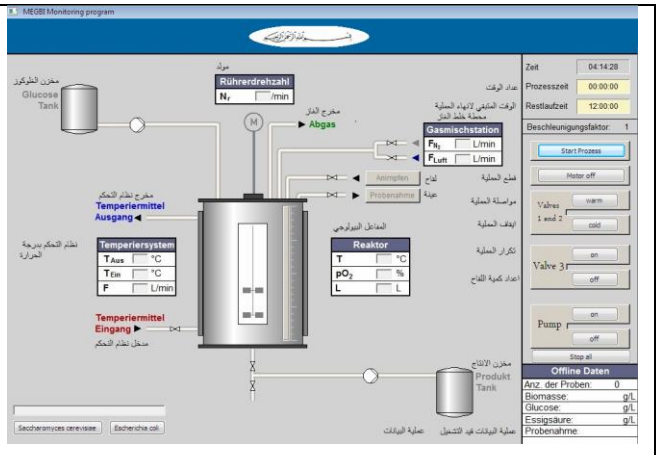
(As increment: simplified monoclonal antibodies production line using the same equipment)

The stainless structure shall be built by TEMO. The instrumentation and special pipes etc. shall be buyed for GE Health or others.

Fig.: Solaris downstream line. The MEGBI-VPP downstream line shall be similar to this.

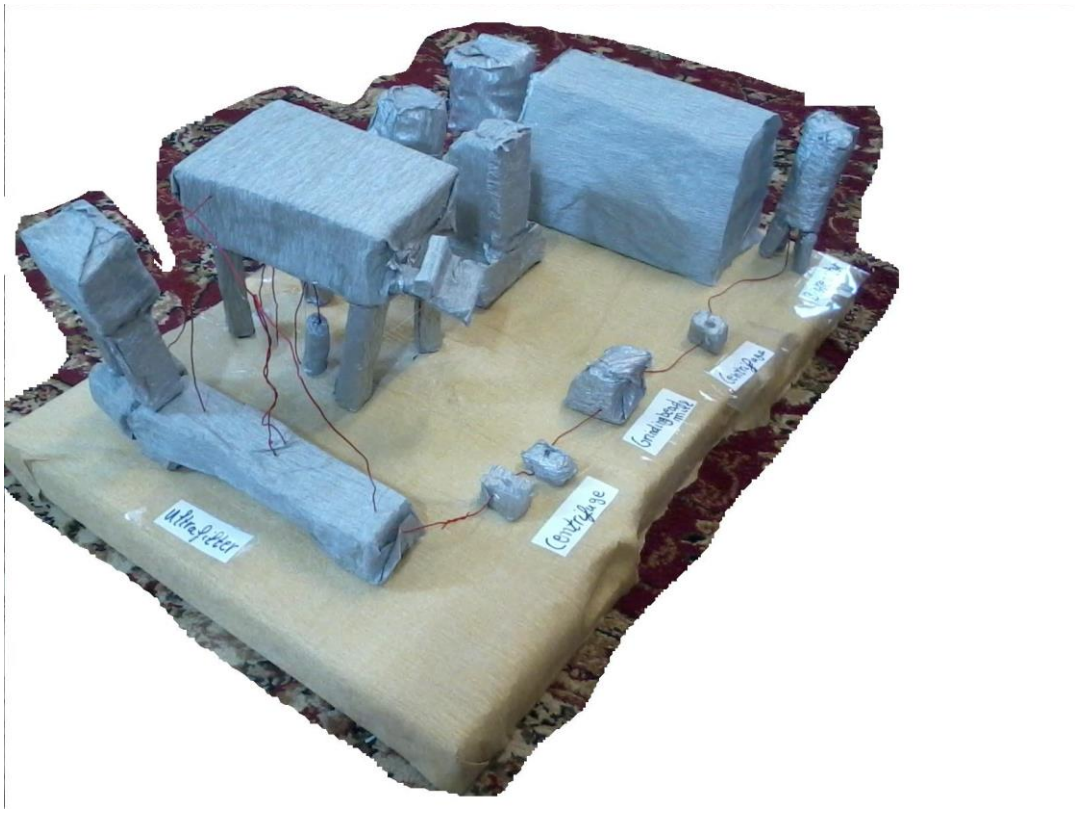
13.2 Automation System

The automation system shall have a C++/phython user interface and a Simatic S7 interface to the sensors/actuators.



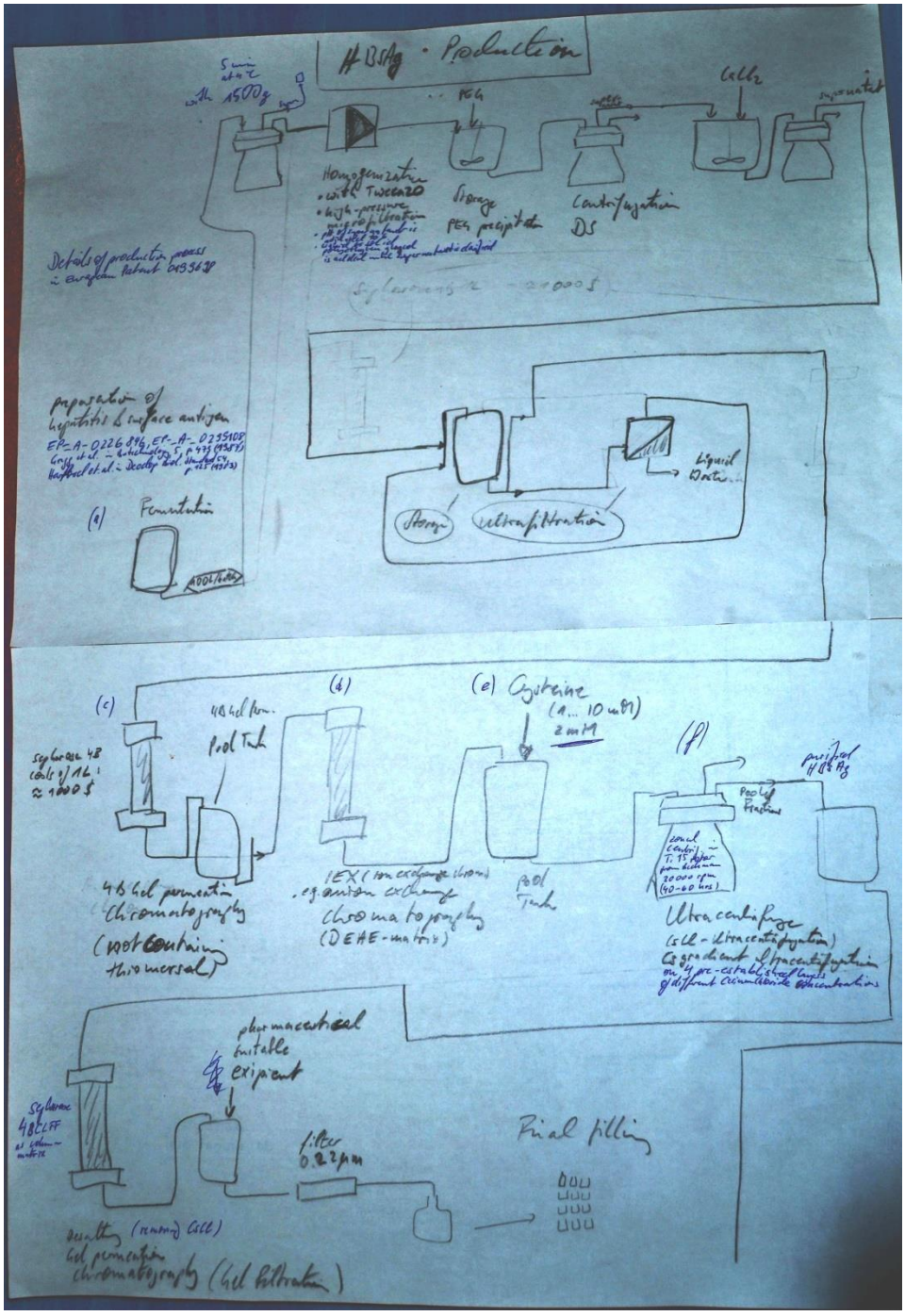
14 System Design

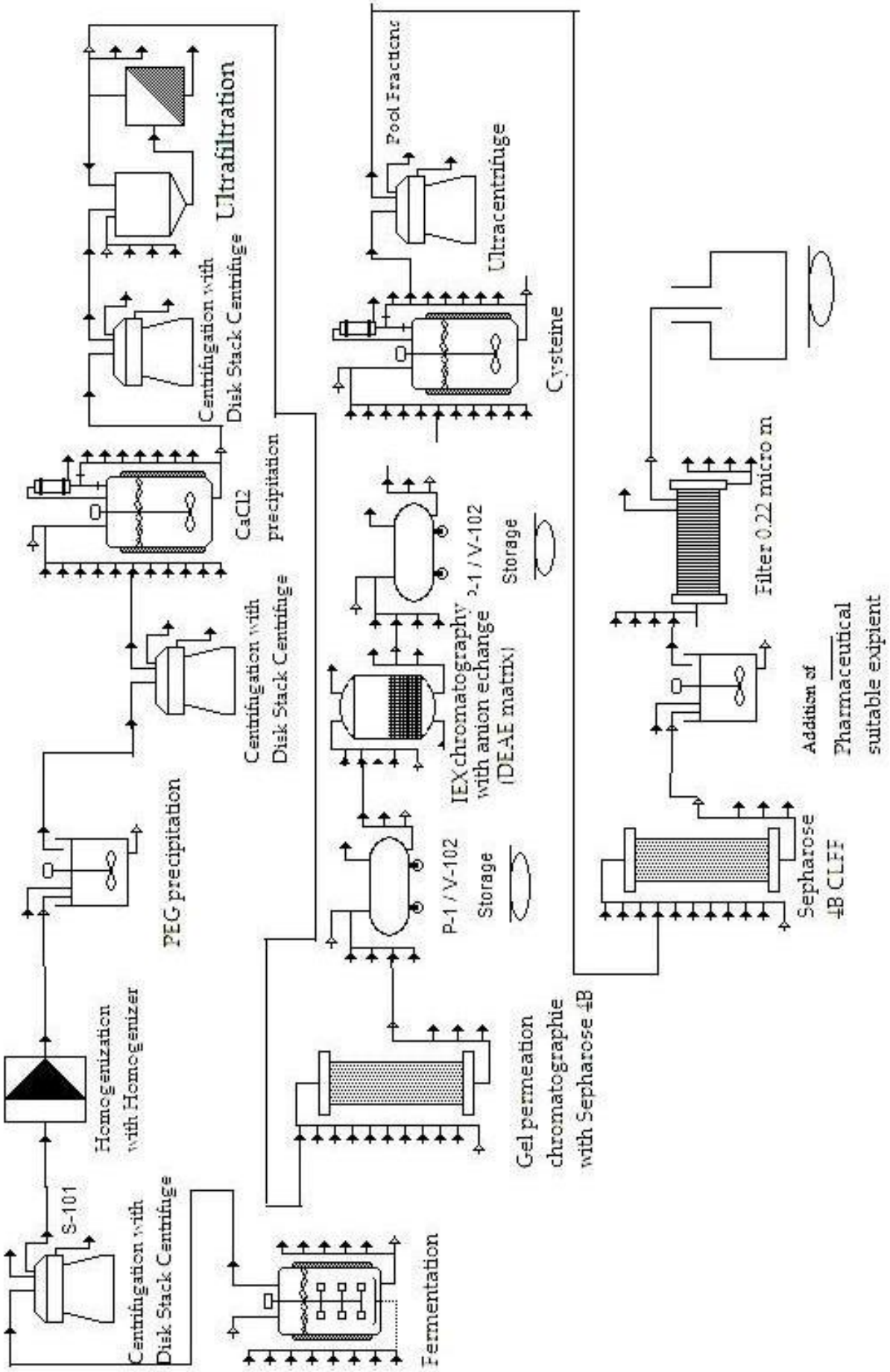
14.1 Mock-up model for the MEBGI vaccine pilot plant



14.2 Integration Overview

Based on the EngerixB patent and the films Bioprocessing Part 2_ Separation _ Recovery and Bioprocessing Part 3_Purification which describe in details the process of Fluorecence Protein Production in E.coli there shall be designed a simplified system for HBSAg vaccine. Using SuperPro SW for describing the design.

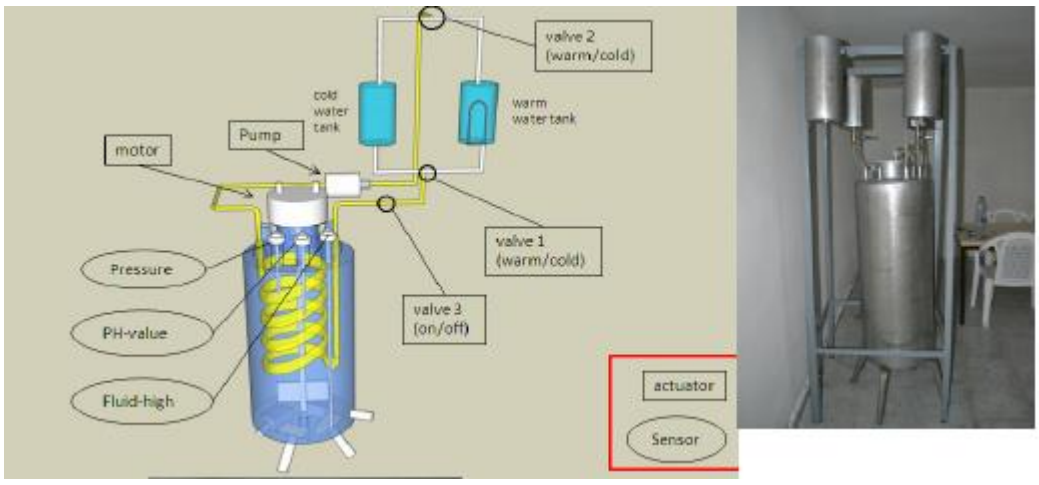



S-101
Centrifugation with Disk Stack Centrifuge
Homogenization with Homogenizer
Fermentation
PEG precipitation
Centrifugation with Disk Stack Centrifuge
CaCl2 precipitation
Centrifugation with Disk Stack Centrifuge
Ultrafiltration
P-1 / V-102
Storage
IEX chromatography with anion echange (DEAE matrix)
P-1 / V-102
Storage
Gel permeation chromatographie with Sepharose 4B
Sepharose 4B CLFF
Ultracentrifuge
Pool Fractions
Cysteine
Filter 0.22 micro m
Addition of Pharmaceutical suitable exipient

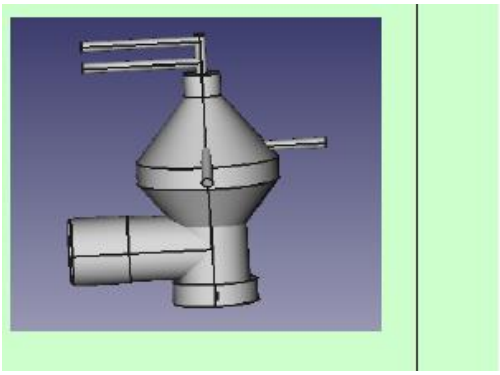
Clean-in-place (CIP)&Steam-in-place (SIP) and intermediate storage	Upstream Processing (USP) – Downstream Processing (DSP)	Automation
 		 

14.3 Fermentation

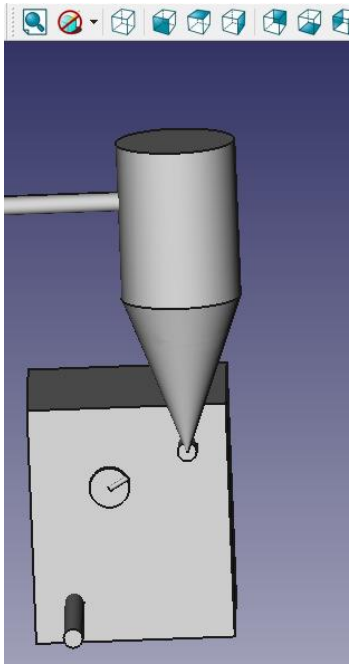
75 L/batch



14.4 Centrifugation with Disc Stack Centrifuge (1)

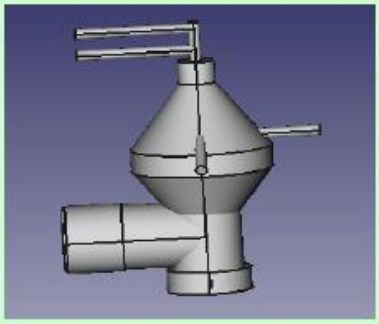


14.5 Homogenization with Homogenizer



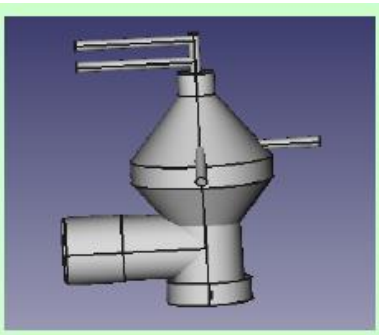
14.6 PEG precipitation

14.7 Centrifugation with Disc Stack Centrifuge (2)



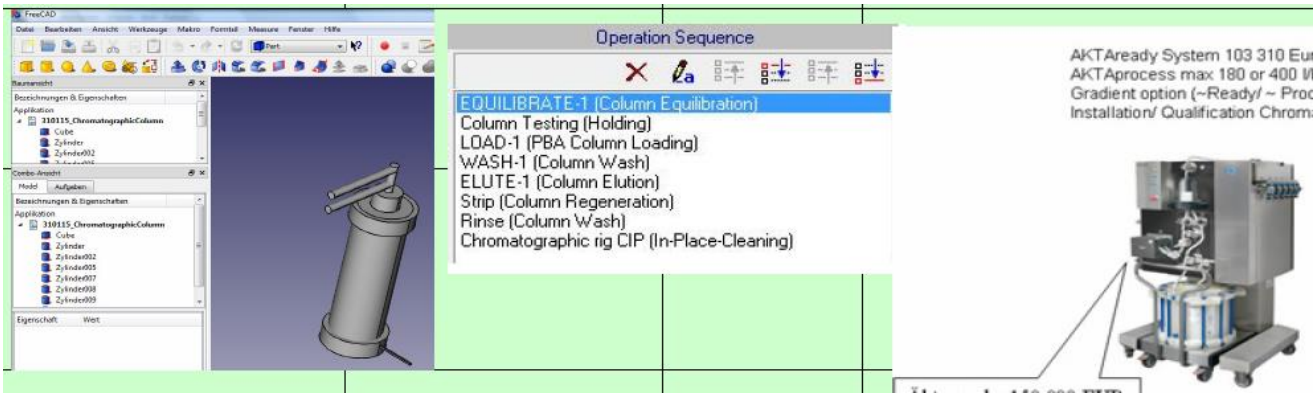
14.8 CaCl2 precipitation

14.9 Centrifugation with Disc Stack Centrifuge (3)

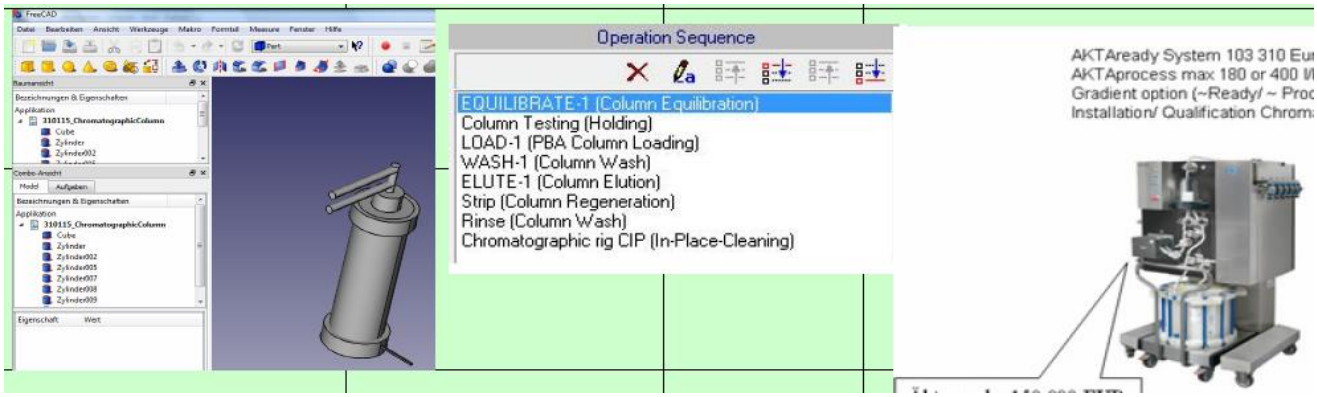


14.10 Ultrafiltration

14.11 Gel permeation chromatography with Sepharose 4B



14.12 IEX chromatography with anion echange (DEAE matrix)



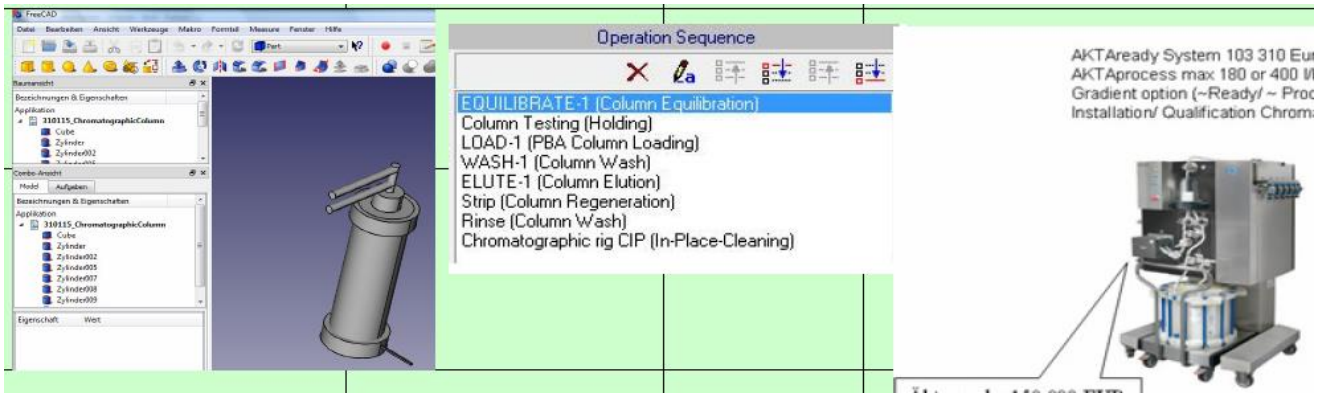
14.13 Cystein addition

14.14 Ultracentrifugation

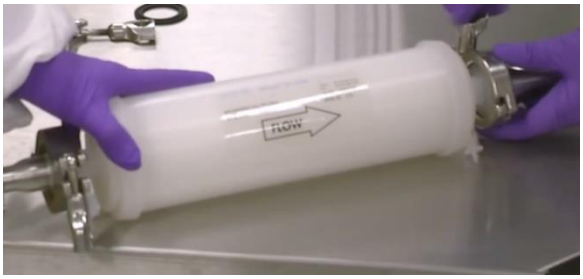
Pooled fractions contain HBSAg

Figure: Alfa Wassermann Ultracentrifuges

14.15 Desalting Gel permeation chromatography with Sepharose 4BCLFF



14.16 Filtering with 0.22 µm filter



15 Detailed Devices Mechanical Design

Done as 6 weeks practical work for 4 students (former planned as master thesis)

بسم الله الرحمن الرحيم

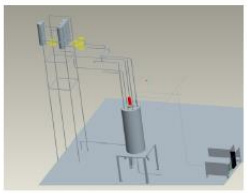
AECENAR
Association for Economical and Technological Cooperation in the Euro-Asian and North-African Region
www.aecenar.com

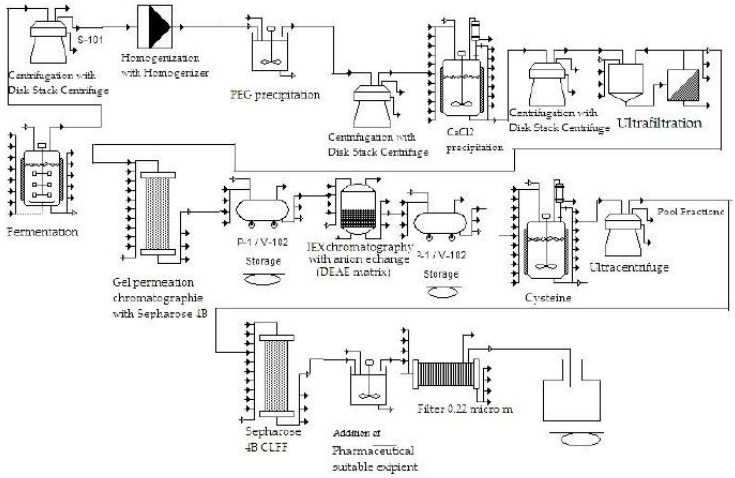
LASeR
Lebanese Association for Scientific Research
الجمعية اللبنانية لدعم البحث العلمي

Hardware & Consulting e.K. - Software
TEMO

Ras Nhache/Batroun - Tripoli, 12th Jan 2015

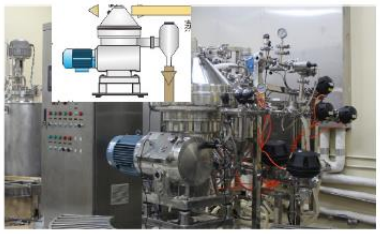
MEGBI Bioreactor





MEGBI-VPP system design:

Downstream System (Purification) from Solaris Group (in this way MEGBI-VPP downstream processing shall be developed)



Disc stack centrifuge from Huading/China

Master Thesis

Modeling and Integration of devices for the vaccine production plant MEGBI-VPP

- Modeling of Homogenizer (3 weeks)
- Modeling of Disc Stack Centrifuge in including CIP/SIP functional elements (mechanics) (3 weeks)
- Modeling Process Scale Gel Permeation and ion exchange chromatographic devices (3 weeks)
- Modeling of Process Scale Ultrafiltration Device (3 weeks)
- Integration of Devices to MEGBI-VPP downstream processing (DSP) unit (2 weeks)
- Documentation (3 weeks)

Keywords: CAD, Tool ProEngineer, Process Scale Homogenizer, Process Scale Centrifugation, Process Scale Protein Chromatography, Biotechnology

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بسم الله الرحمن الرحيم

Project: MEGBI-VPP (Detailed Devices Mechanical Design for 3rd MEGBI-VPP Report)

Report of Practical Work Jun/Jul 2015 (6 Weeks), Final version: 19 Aug 2015

Authors: Jihad Samarji, ZaherChendeb, Ibrahim Zaaroura, FadiYahya

OBJECTIVE AND PURPOSE OF THE ASSOCIATION:

The association is committed to the promotion of international cooperation in the economic and scientific fields in order to achieve the idea of international understanding and a closer relationship between institutions the Middle East, in Europe and its neighbors. To Download the AECENAR flyer as pdf please click here: AECENAR Brochure 2014

INSTITUTE:

MEGBI - Middle East Genetics and Biotechnology Institute

PROJECT:

MEGBI-VPP / DNA Vaccine Pilot Plant, Recombinant Vaccine Technology / Biotechnological Upstream & Downstream Processing Hepatitis B DNA Vaccine Pilot Plant

PROJET CURRENT TASKS:

Modelling of homogenizer

Modelling of Disc Stack Centrifuge in including CIP/SIP functional elements (mechanics)

Modelling Process Scale Gel Permeation and Ion Exchange Chromatography Devices

Modelling of Process Scale Ultrafiltration Device

Integration of Devices to MEGBI-VPP Downstream processing (DSP) unit

Documentation

SKILLS NEEDED:

CAD, Process Scale Homogenizer, Process Scale Centrifugation, Process Scale Protein Chromatography

SOFTWARE USED:

FREECAD (+ Python MACROS), Microsoft OFFICE, Adobe Suite, Windows OS / Linux OS

15.1 Demonstration and Modelling of the Ultracentrifuge.

"To Separate the High Density from the Low Density Molecules, We Use The Ultracentrifuge." –Jihad Samarji

Figure 20.1 PKII Ultra Centrifuge

15.1.1 DEVICE DETAILS & SPECIFICATIONS

The **ultracentrifuge** is a centrifuge optimized for spinning a rotor at very high speeds, capable of generating acceleration as high as 2 000 000 g (approx. 19 600 km/s²). There are two kinds of ultracentrifuges, the preparative and the analytical ultracentrifuge. Both classes of instruments find important uses in molecular biology, biochemistry, and polymer science.

In our Case we're using the Preparative Ultracentrifuge

Preparative ultracentrifuges are available with a wide variety of rotors suitable for a great range of experiments. Most rotors are designed to hold tubes that contain the samples. *Swinging bucket rotors* allow the tubes to hang on hinges so the tubes reorient to the horizontal as the rotor initially

accelerates. *Fixed angle rotors* are made of a single block of material and hold the tubes in cavities bored at a predetermined angle.

In our Case we're using the Zonal Rotor

Zonal rotors are designed to contain a large volume of sample in a single central cavity rather than in tubes. Some zonal rotors are capable of dynamic loading and unloading of samples while the rotor is spinning at high speed.

Preparative rotors are used in biology for pelleting of fine particulate fractions, such as cellular organelles (mitochondria, microsomes, and ribosomes) and viruses. They can also be used for gradient separations, in which the tubes are filled from top to bottom with an increasing concentration of a dense substance in solution. Sucrose gradients are typically used for separation of cellular organelles. Gradients of caesium salts are used for separation of nucleic acids. After the sample has spun at high speed for sufficient time to produce the separation, the rotor is allowed to come to a smooth stop and the gradient is gently pumped out of each tube to isolate the separated components. In our Case we're using it to produce anti-bodies

We Need To Design a Density Gradient Ultracentrifugation System for large scale and pilot scale downstream processing of viral vaccines and virus like particles.

	Air Drive
Models Available	KII and PKII
Process Air	2.83 m^3/min
Process Cooling	4.5 °C, 4 l/min
Electrical Supply	15A, 1ph 230V
Environmental Conditions	0-40°C, RH 85%
Clean Room Classifications	Class B,C,D
Bio-containment	Up to BL3
System Footprint	2140 x 1330 (W x D)
Height Requirements	295 cm (PKII 220 cm)
System Weight	1270 kg (PKII 1204 kg)

Table 1.1 Ultracentrifuge KII&PKII specifications

So we're going to design something close to the ALFA WASSERMAN KII & PKII Continuous Flow Ultracentrifuge model. See figure 1.1 and 1.2

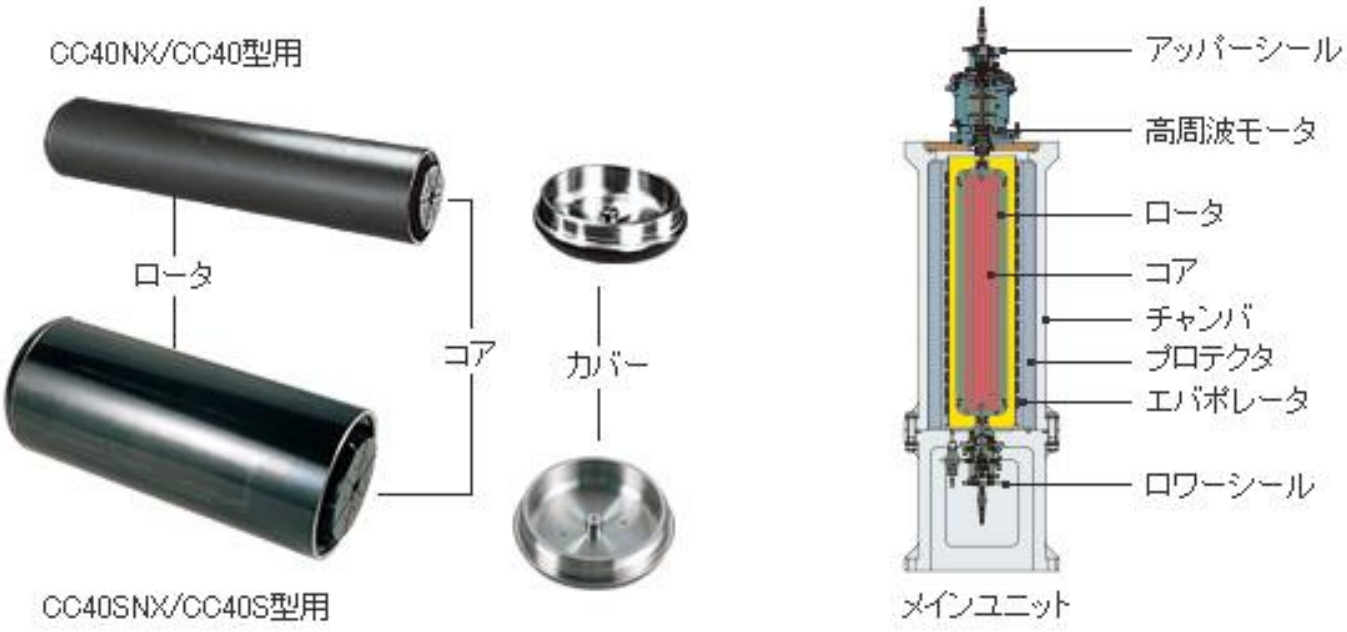
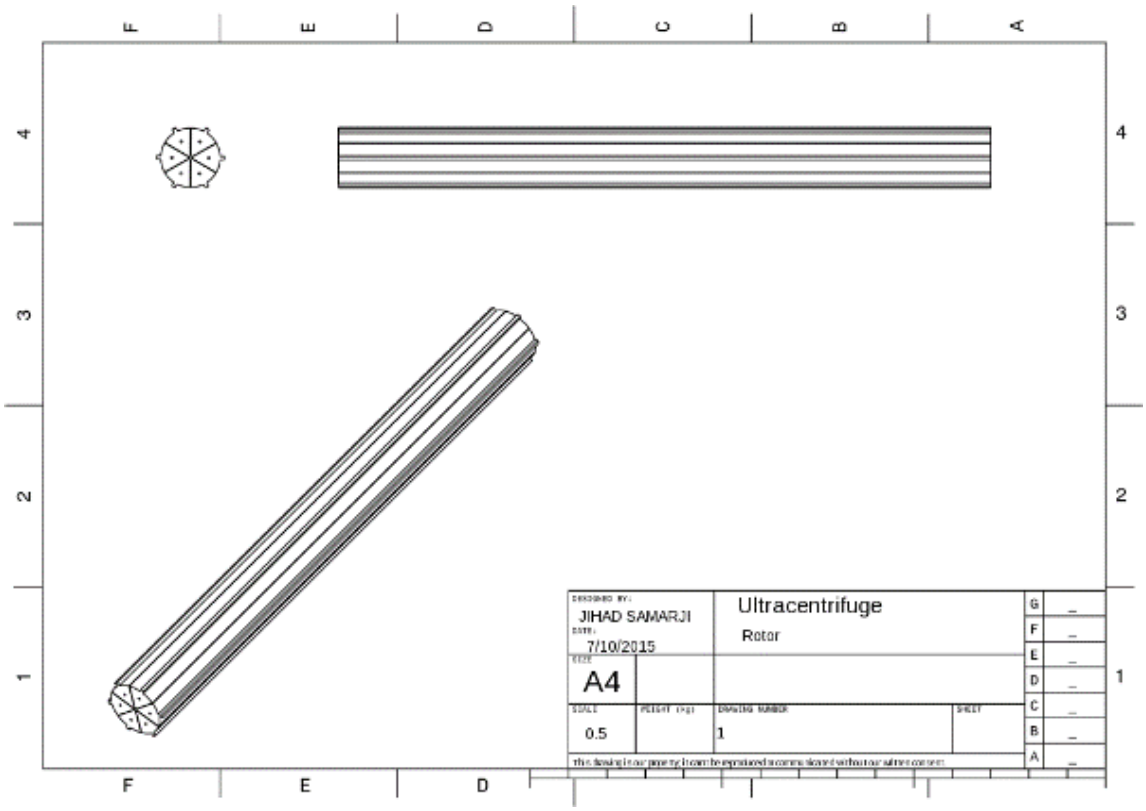


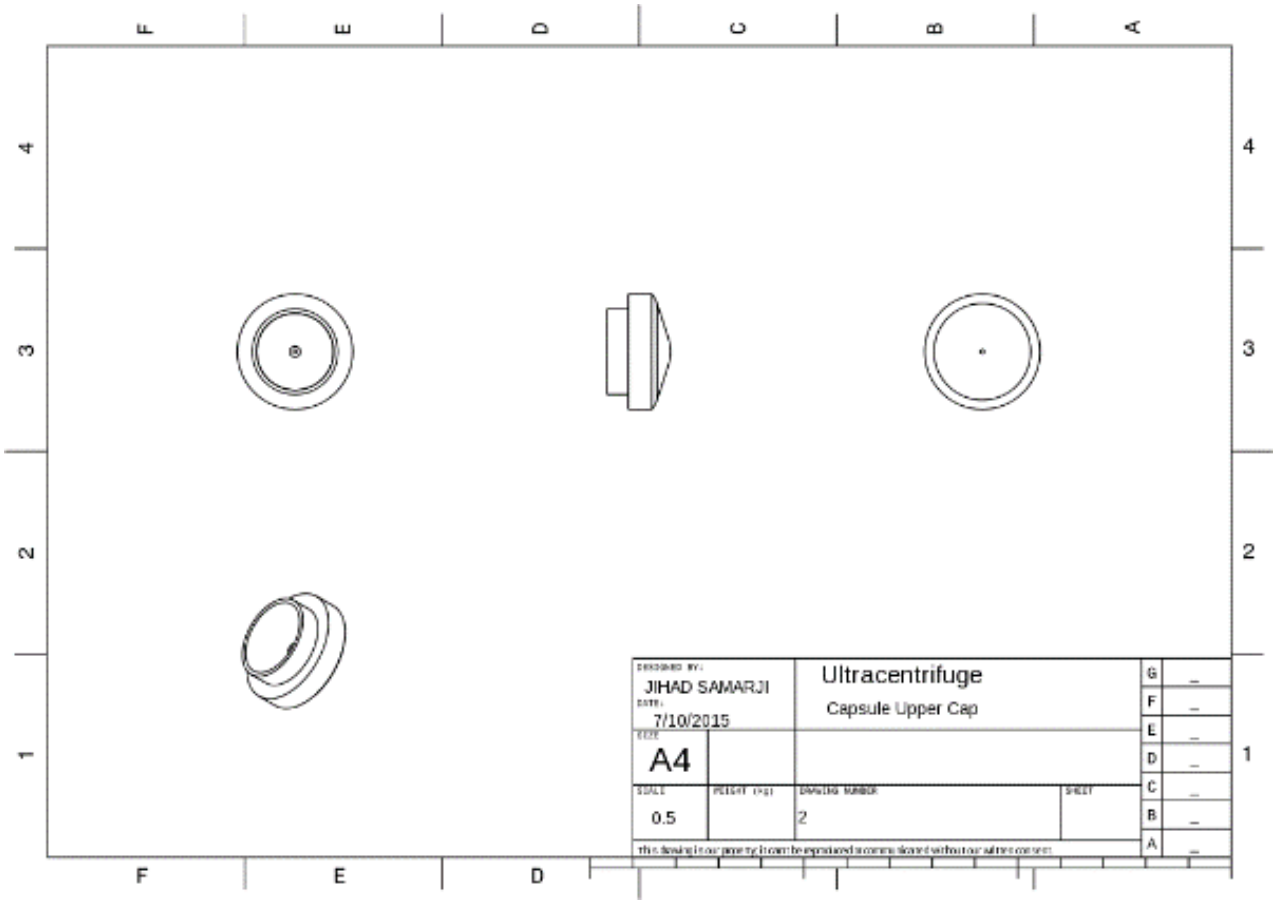
Figure 1.2 Ultracentrifuge annotation in Chinese

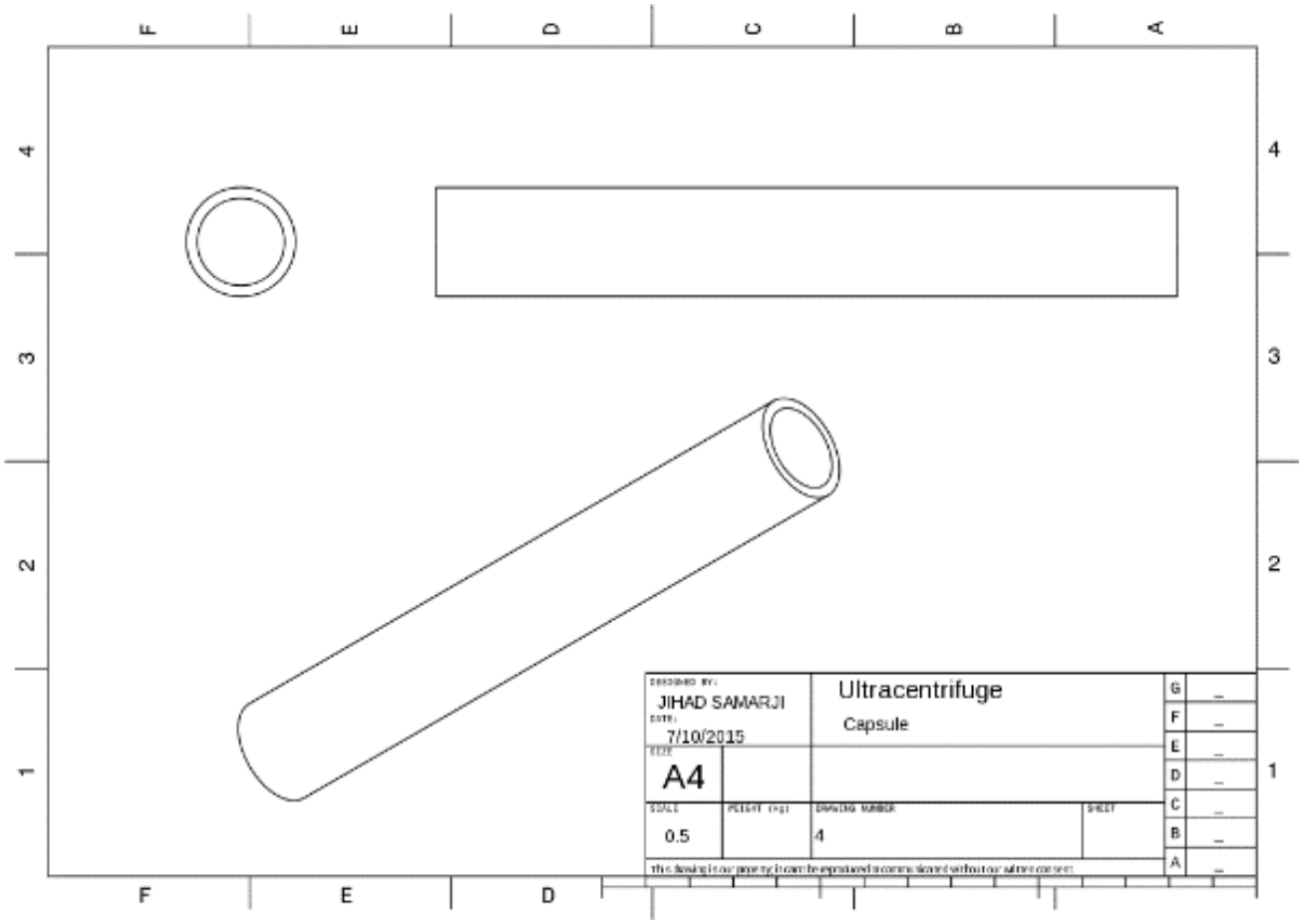
The Ultracentrifuge can be used For Uranium Enrichment which makes it very dangerous and Complicated, Therefore gaining information about this particular machine is very limited. This is by far the clearest picture describing the Ultracentrifuge on the internet.

In fact, searching for the Keyword "Ultracentrifuge" on Google makes you suspicious for Global Terrorism. BE CAUTIOUS

15.1.2 PART DIMENSIONS & DESIGN



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JIHAD SAMARJI
DATE:
7/10/2015
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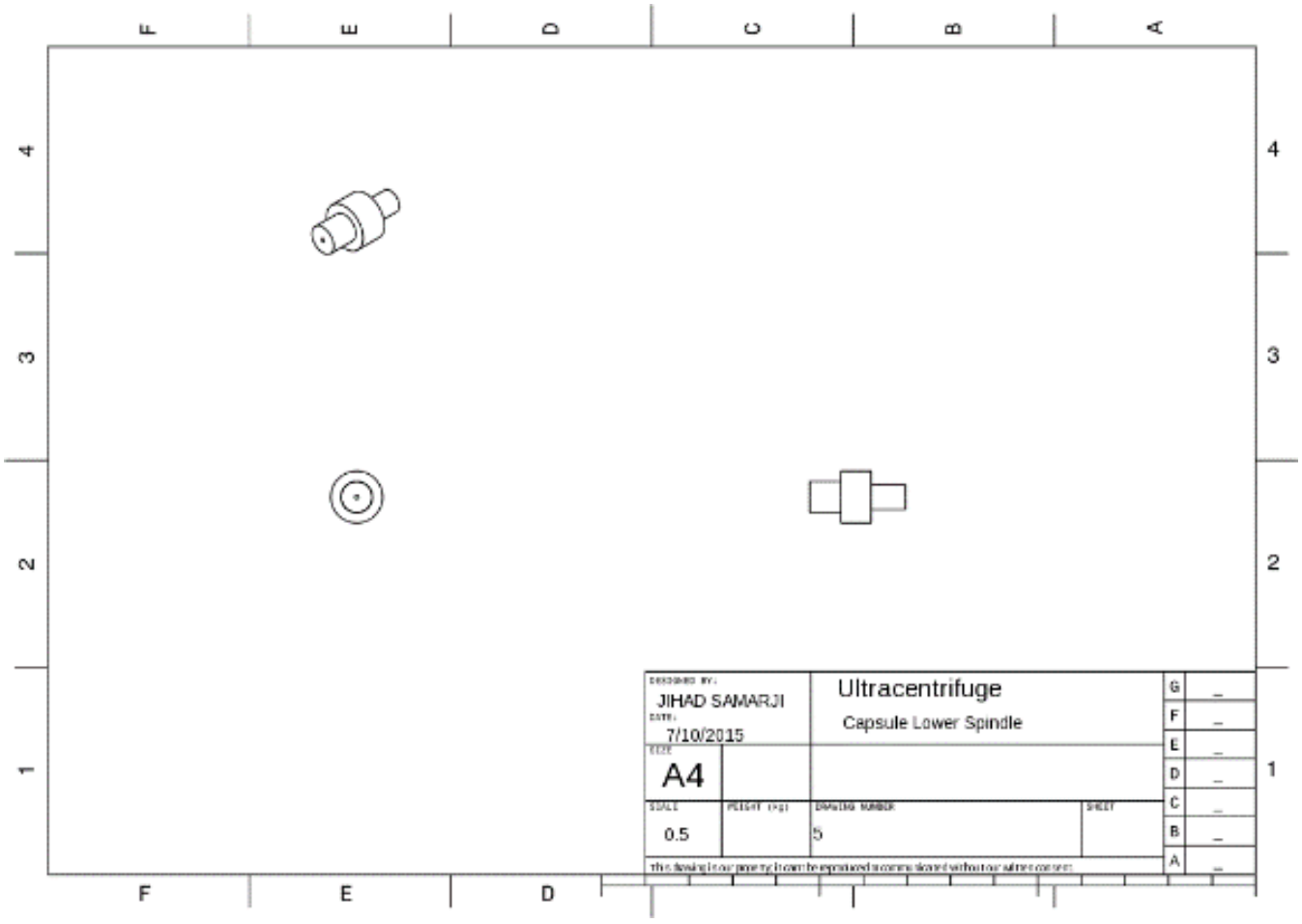
Ultracentrifuge
Capsule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DATE:
7/10/2015
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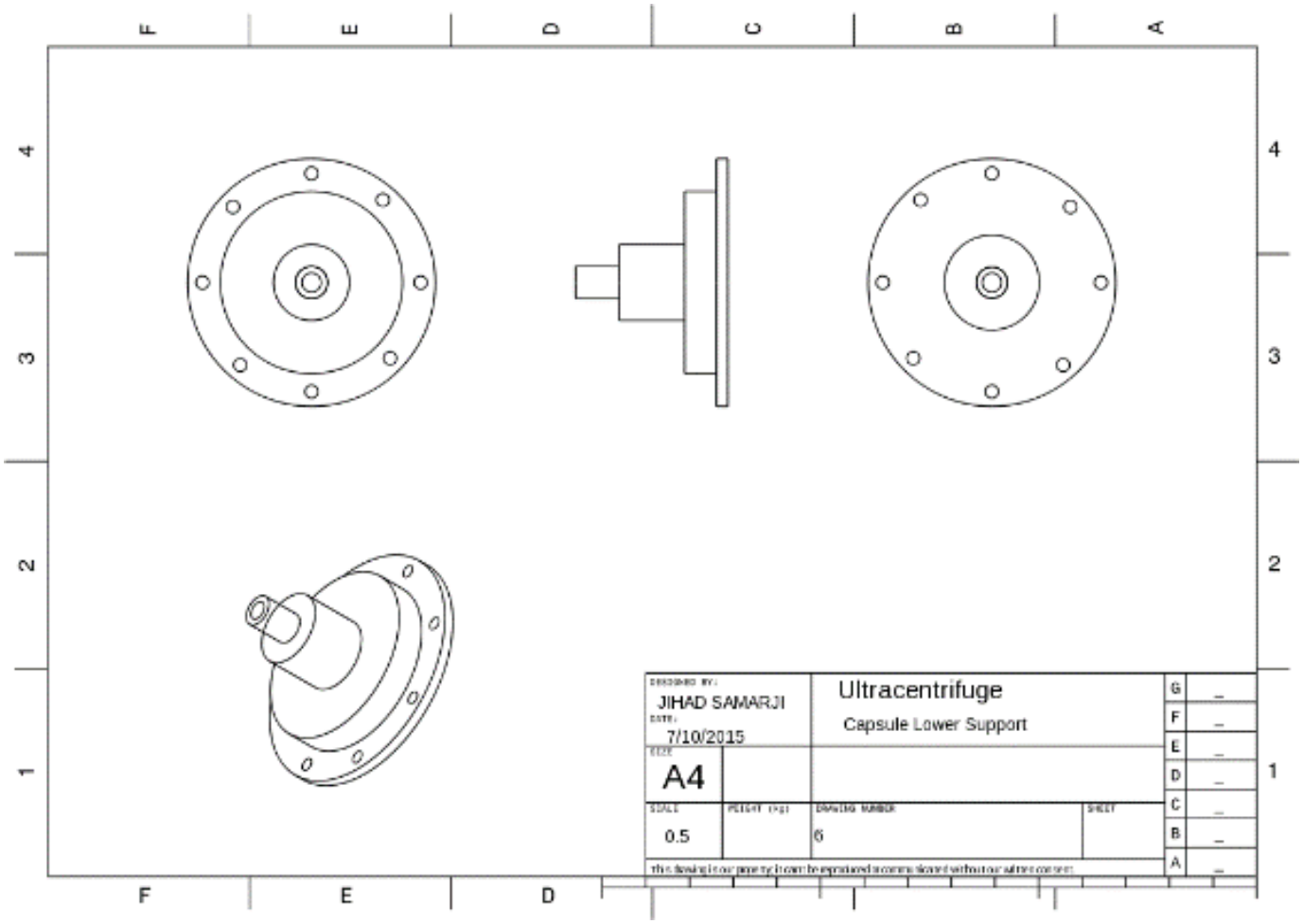
Capsule Lower Spindle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SCALE
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WEIGHT (kg)
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G
F
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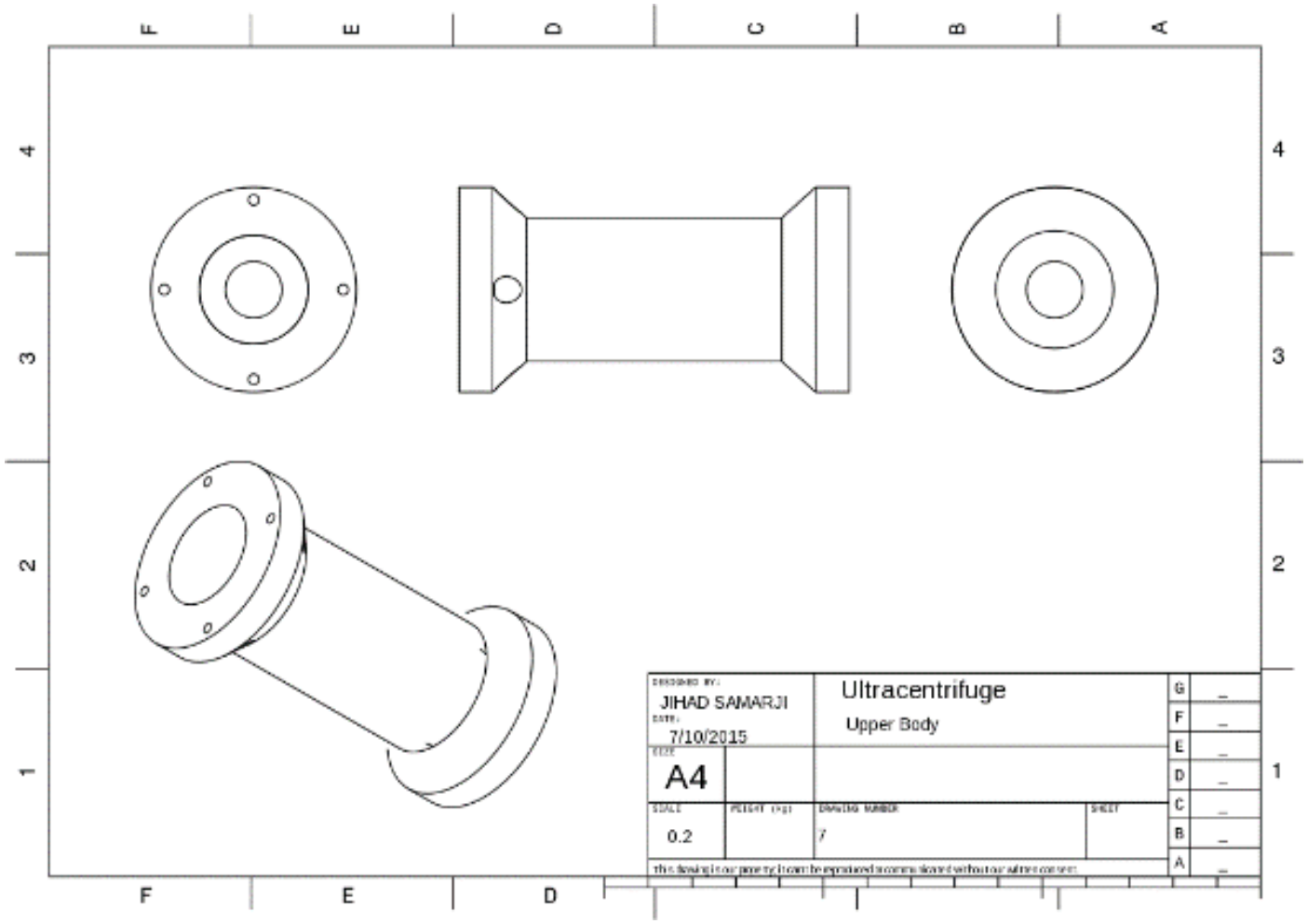
A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7/10/2015
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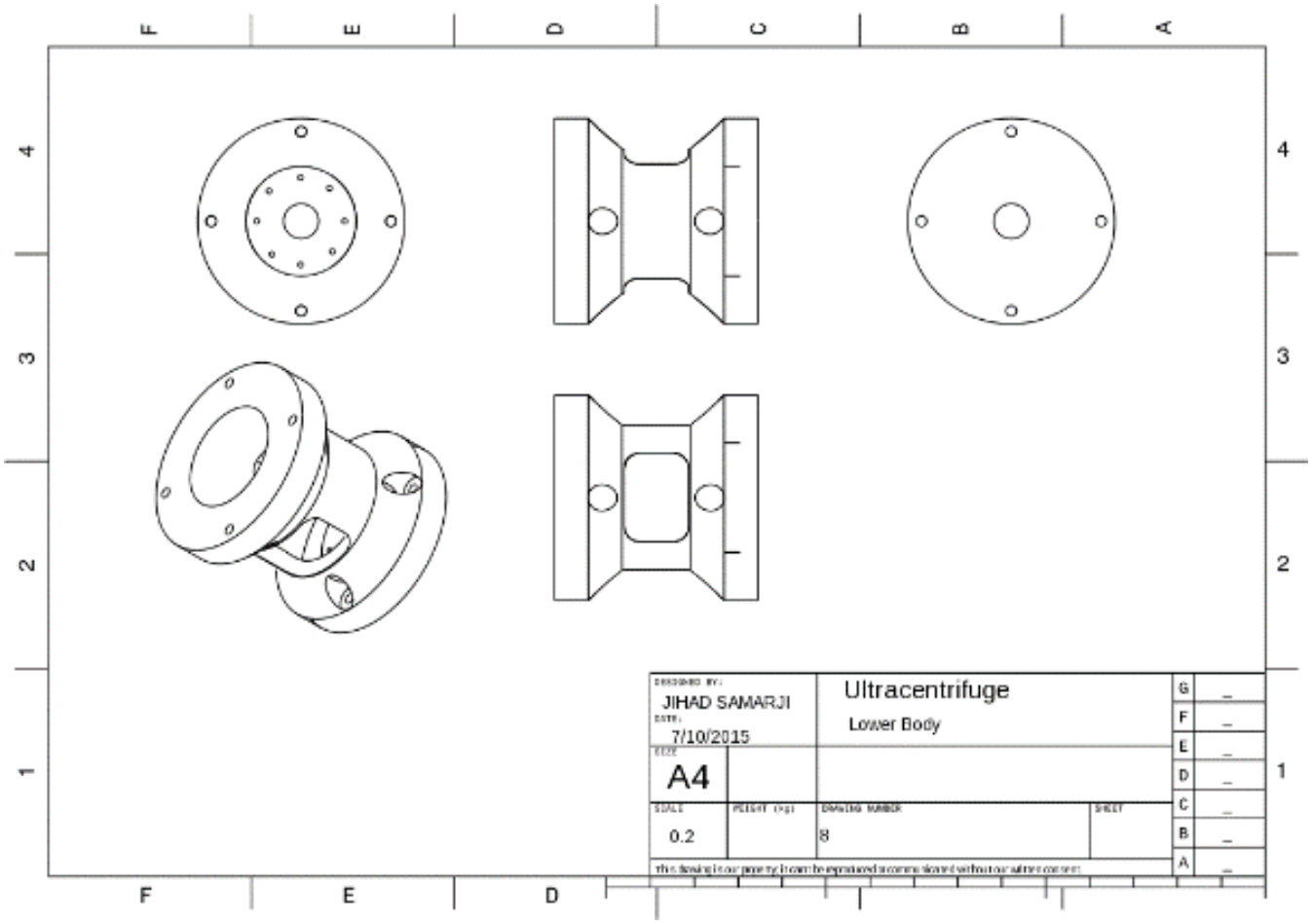
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WEIGHT (kg)
DRAWING NUMBER
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SHEET
Ultracentrifuge
Lower Body
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C
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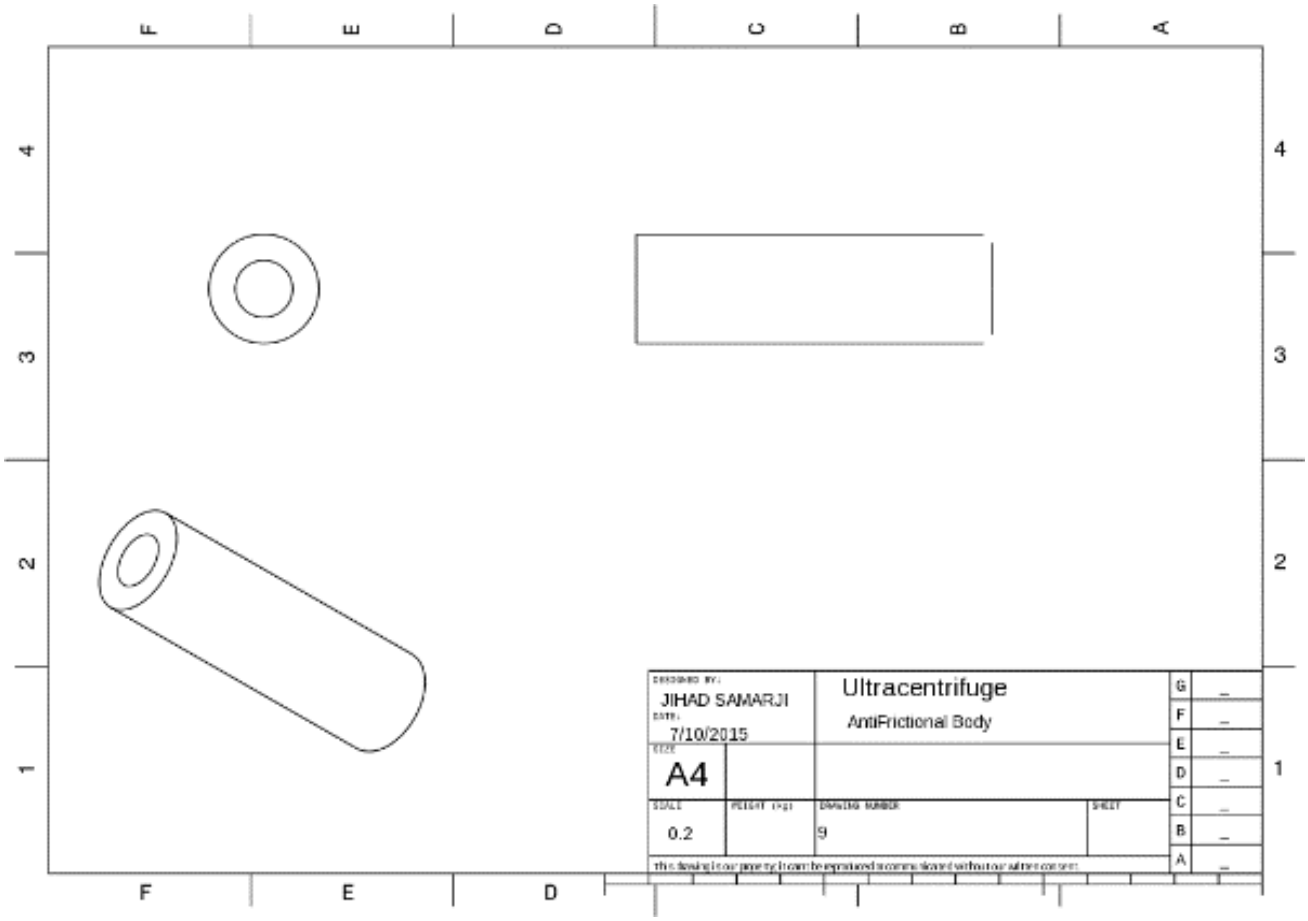
A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AntiFrictional Body
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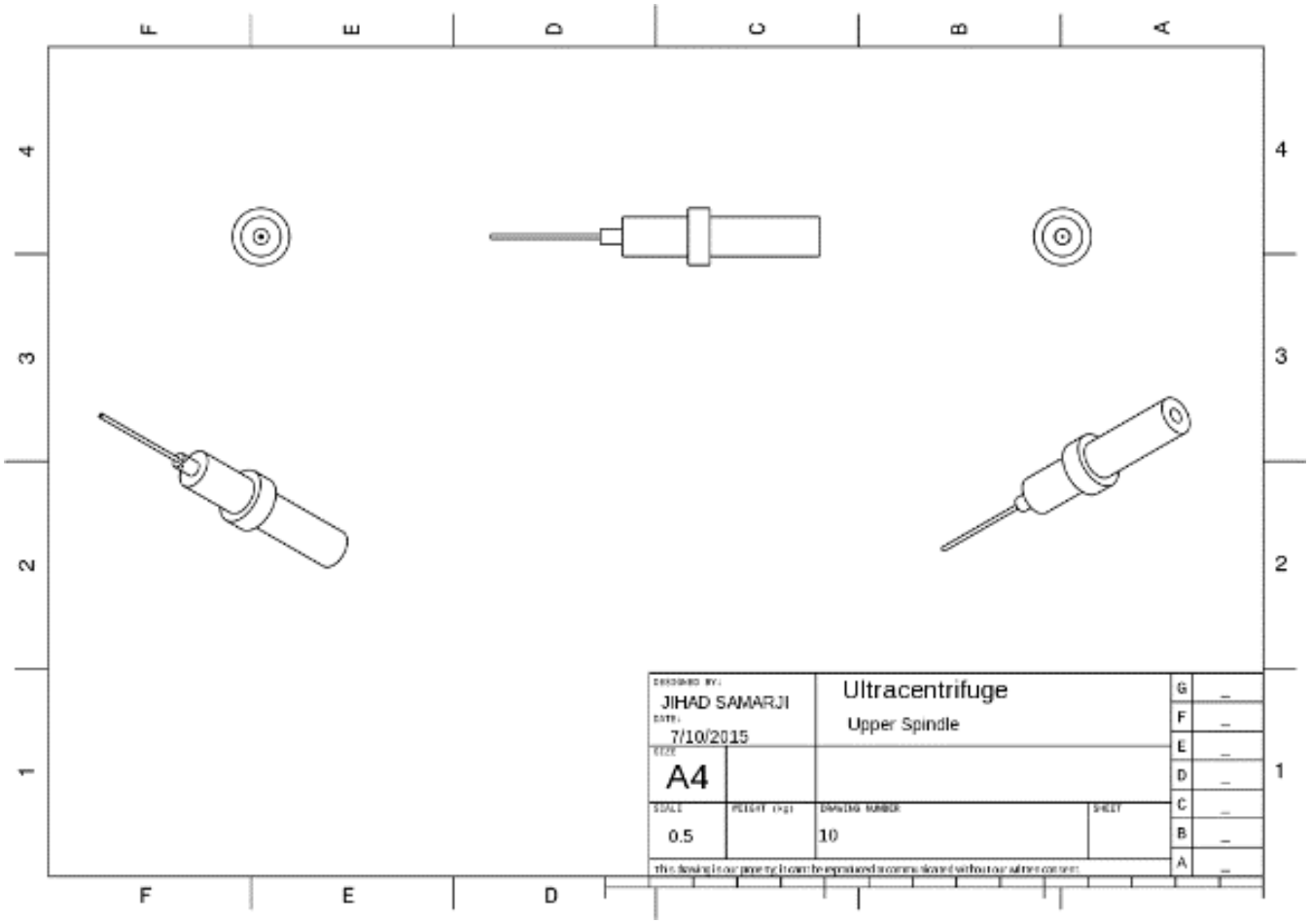
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Upper Spindle
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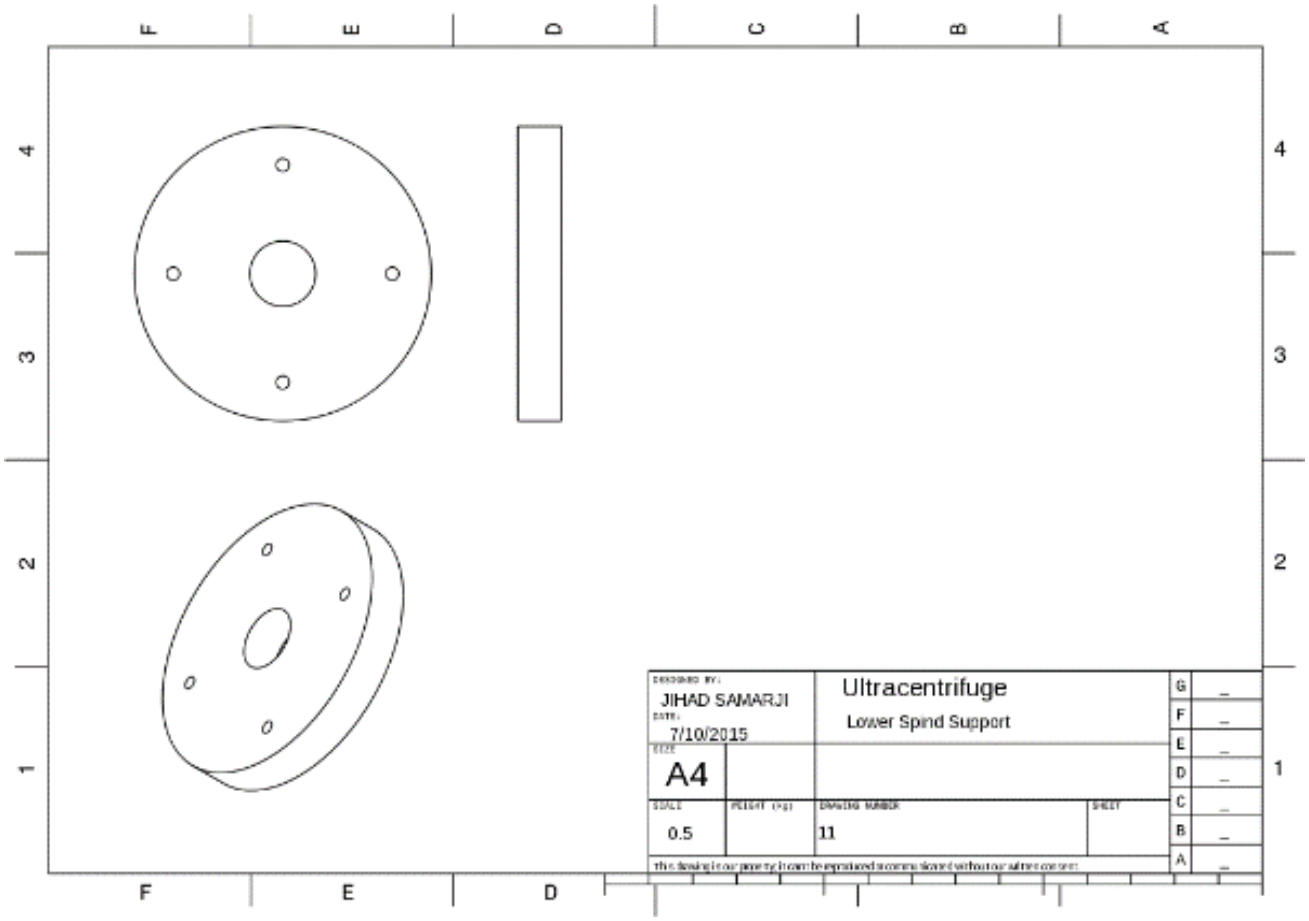
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11
G
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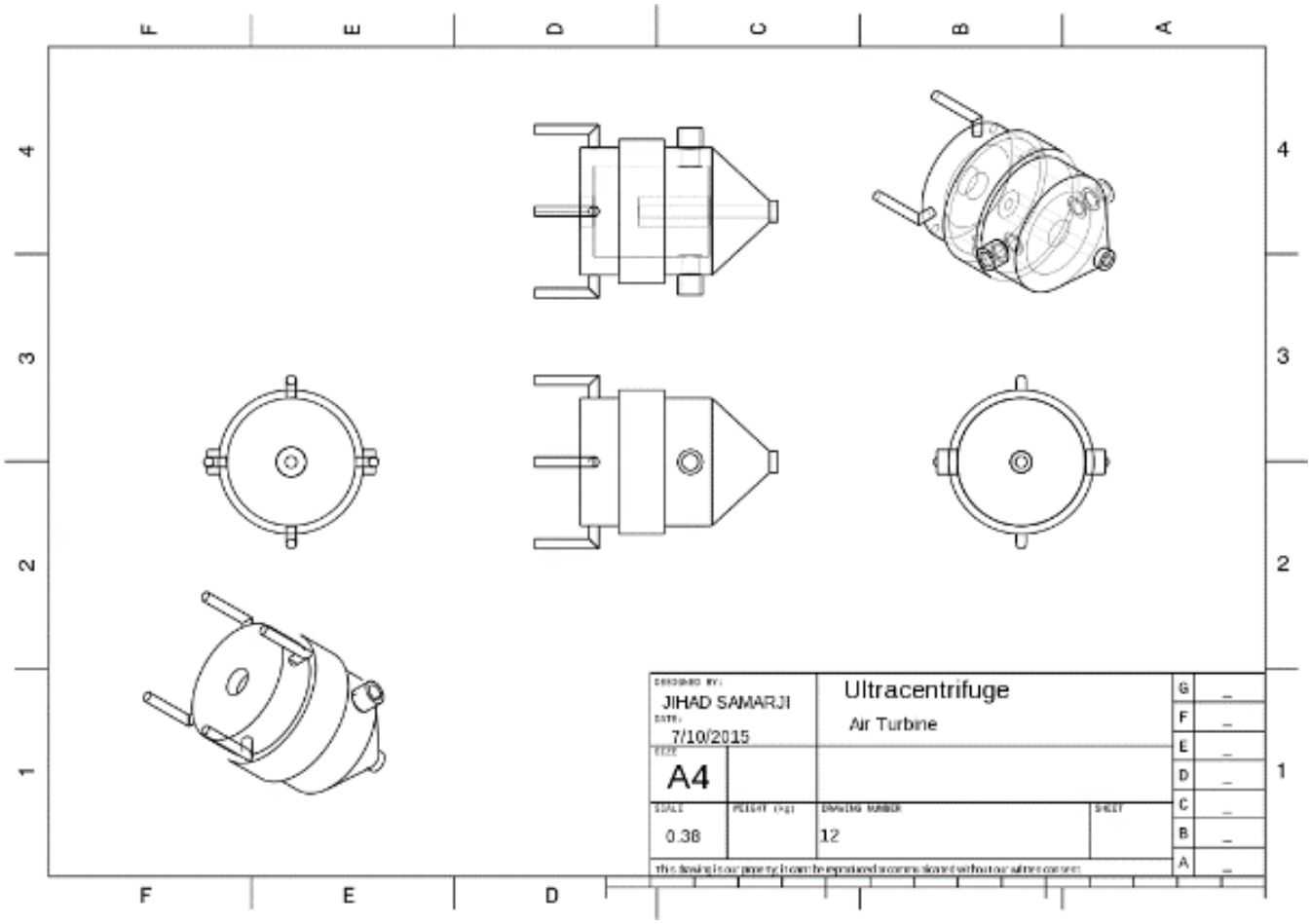
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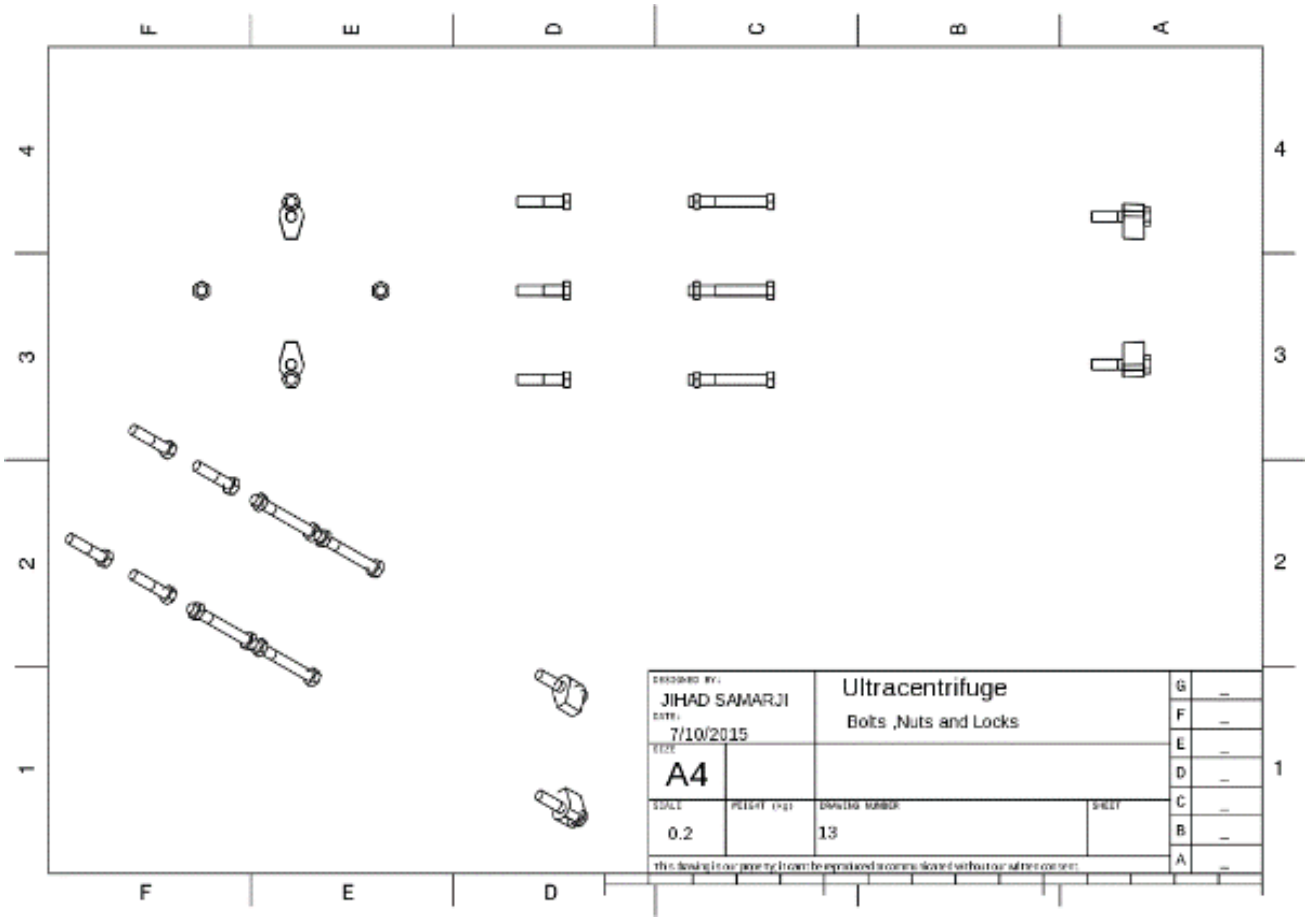
Air Turbine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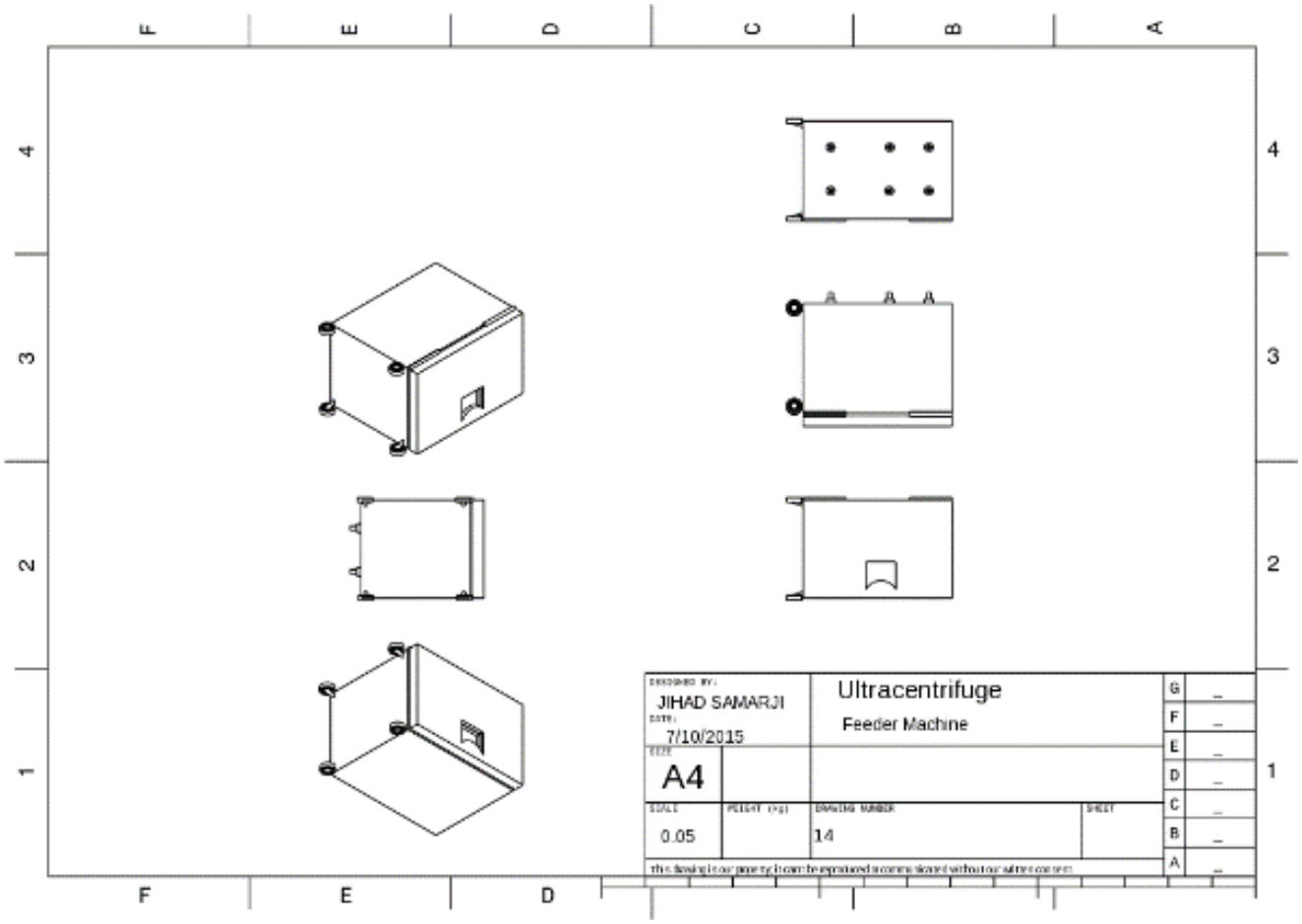
Bolts ,Nuts and Locks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D
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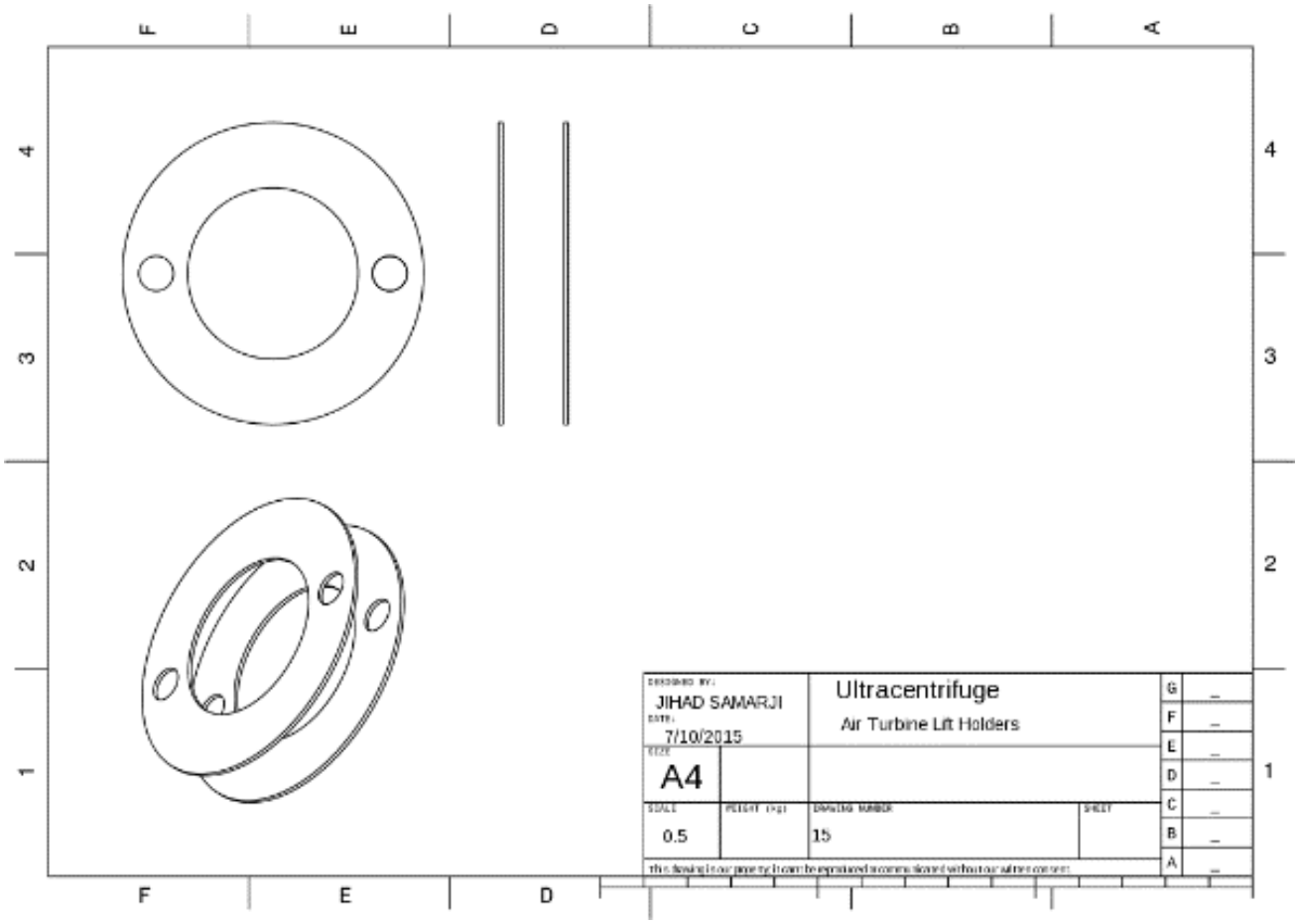
B
A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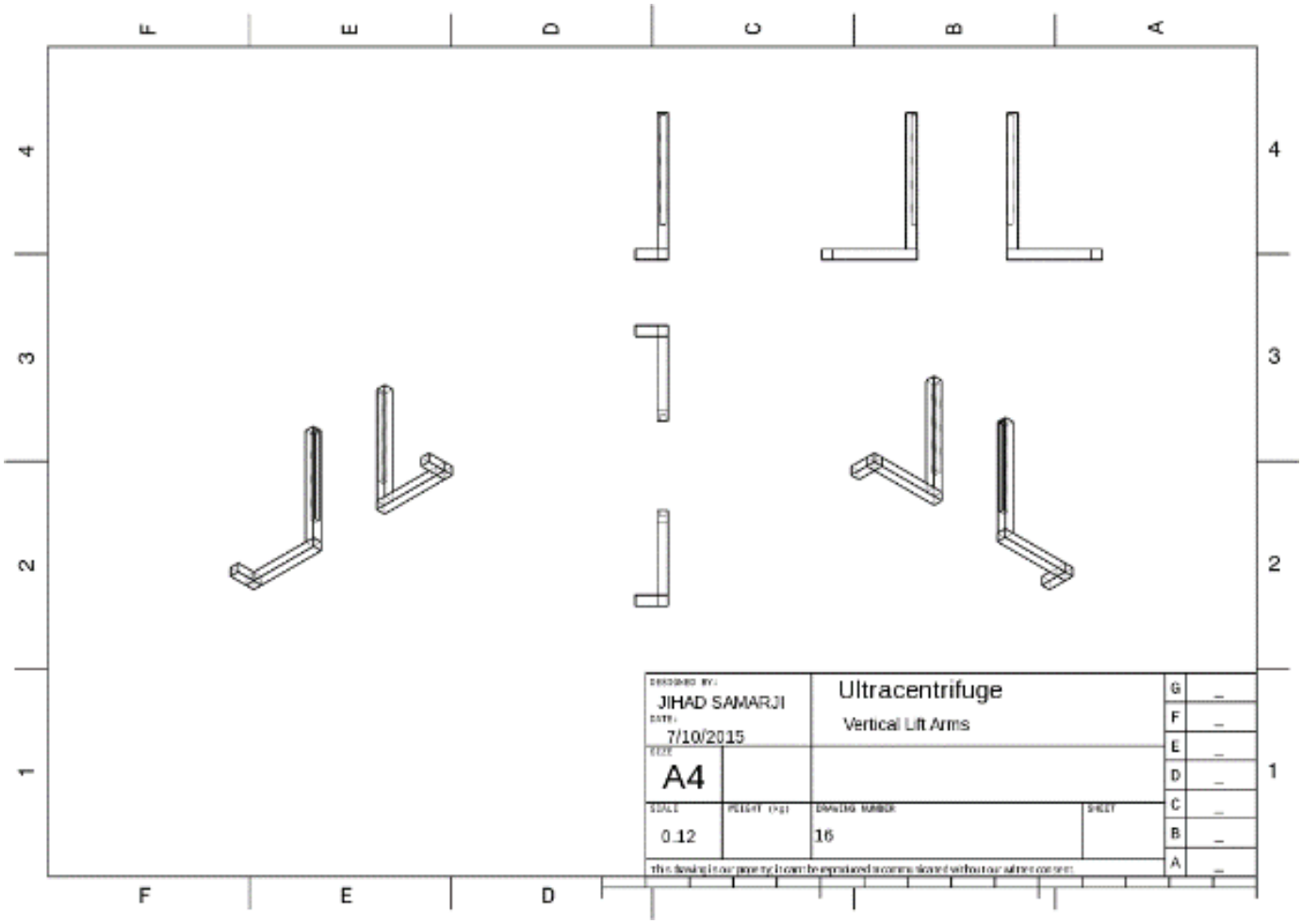
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SCALE
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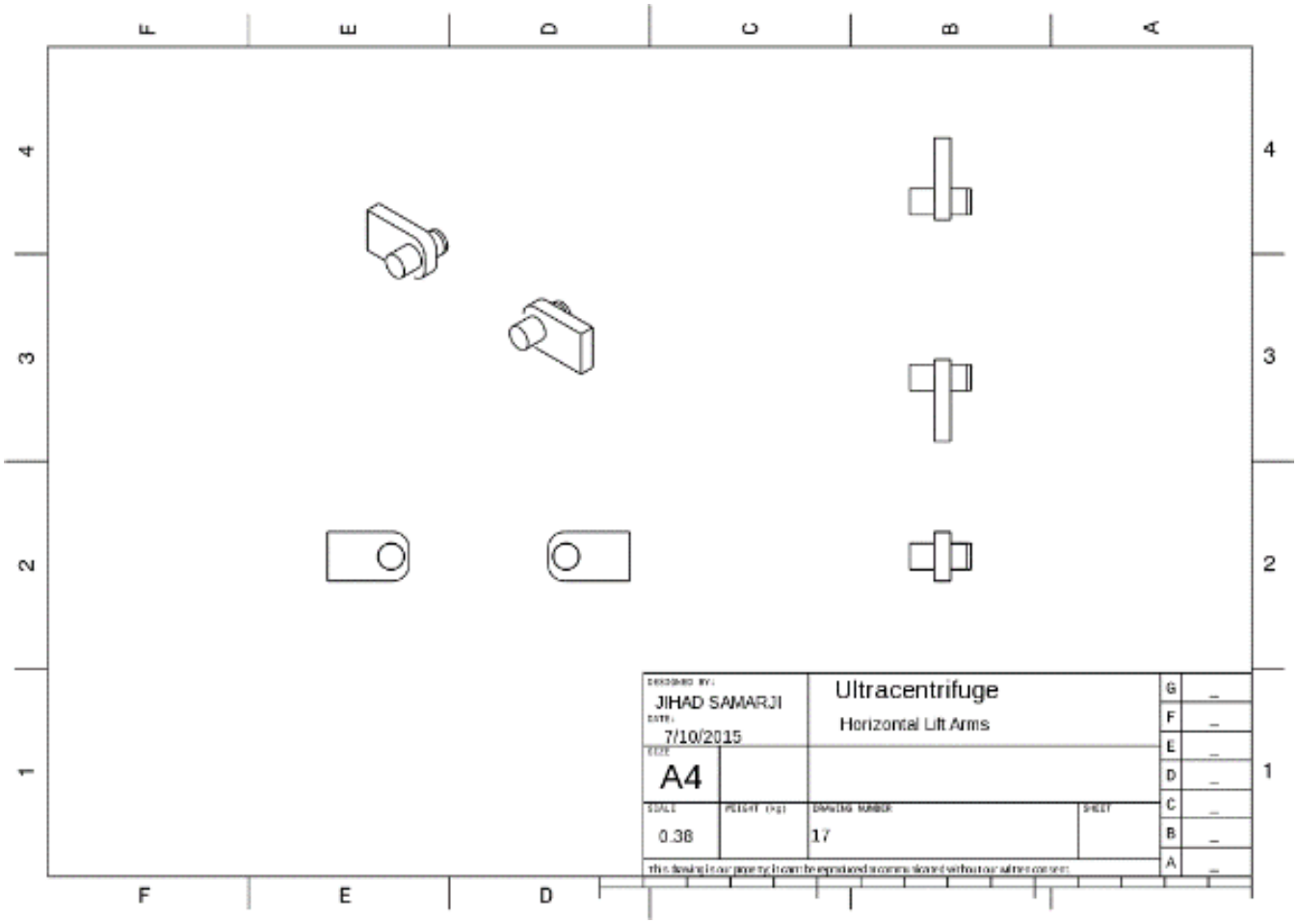
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Ultracentrifuge
Horizontal Lift Arms
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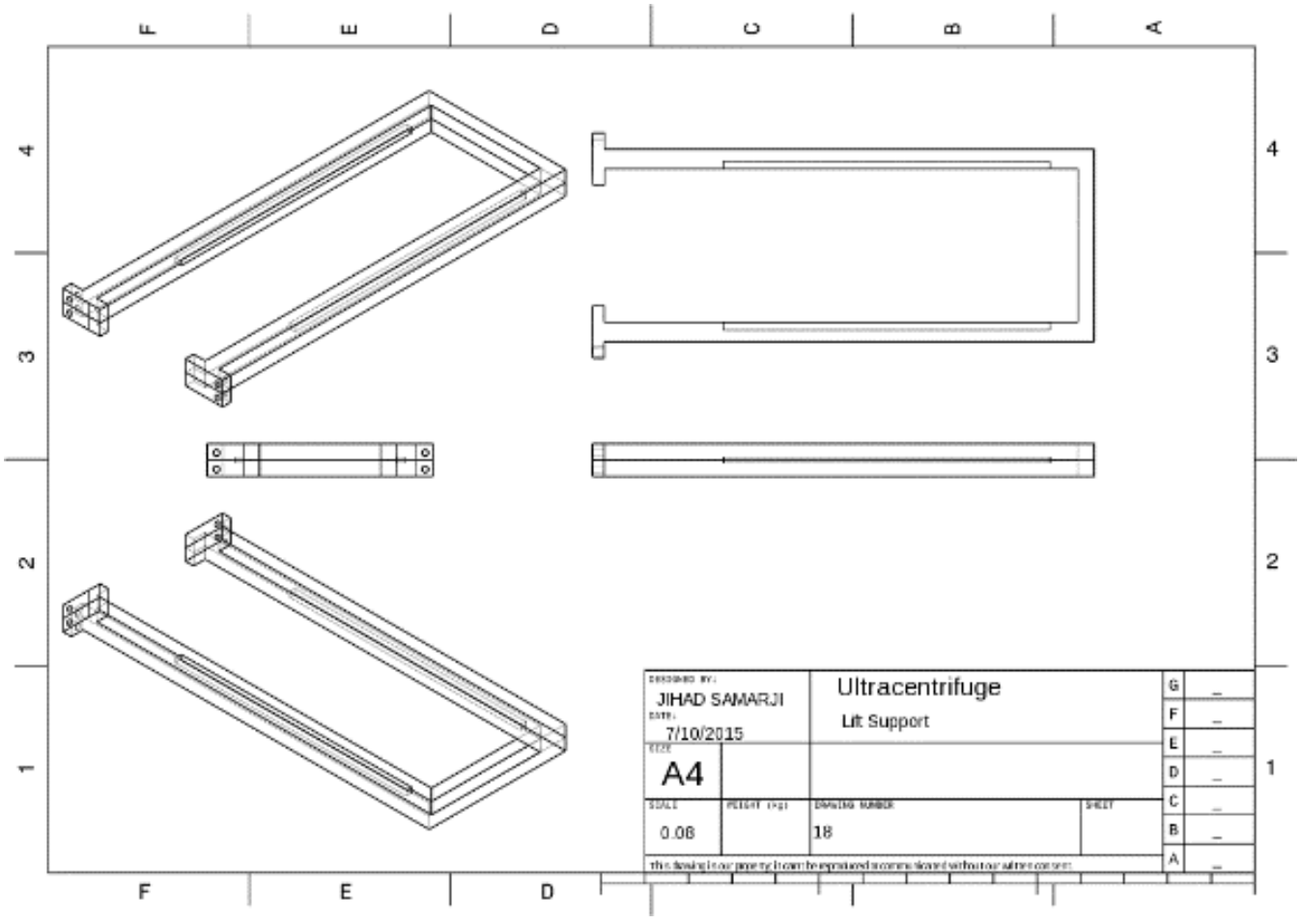
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Lift Support
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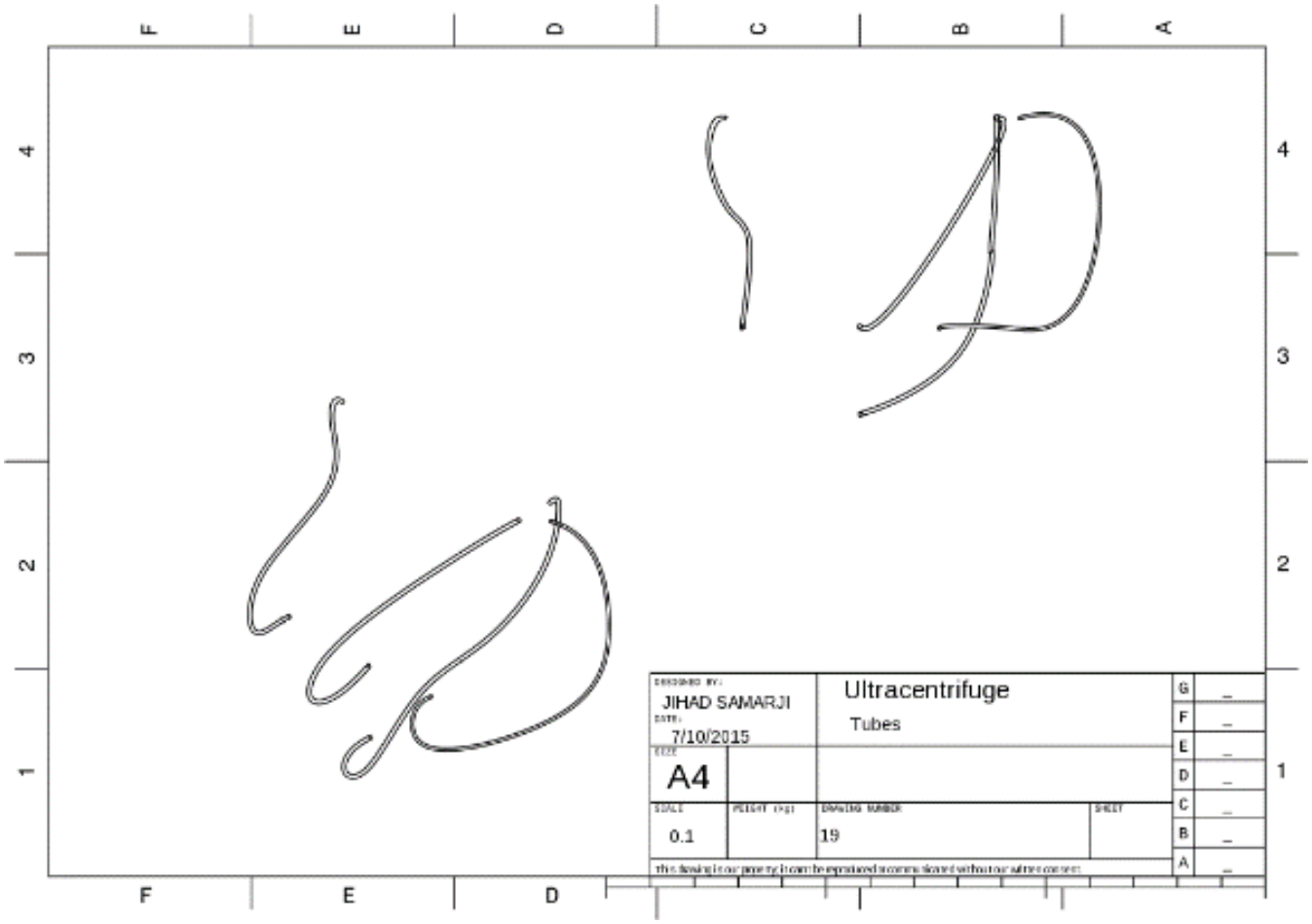
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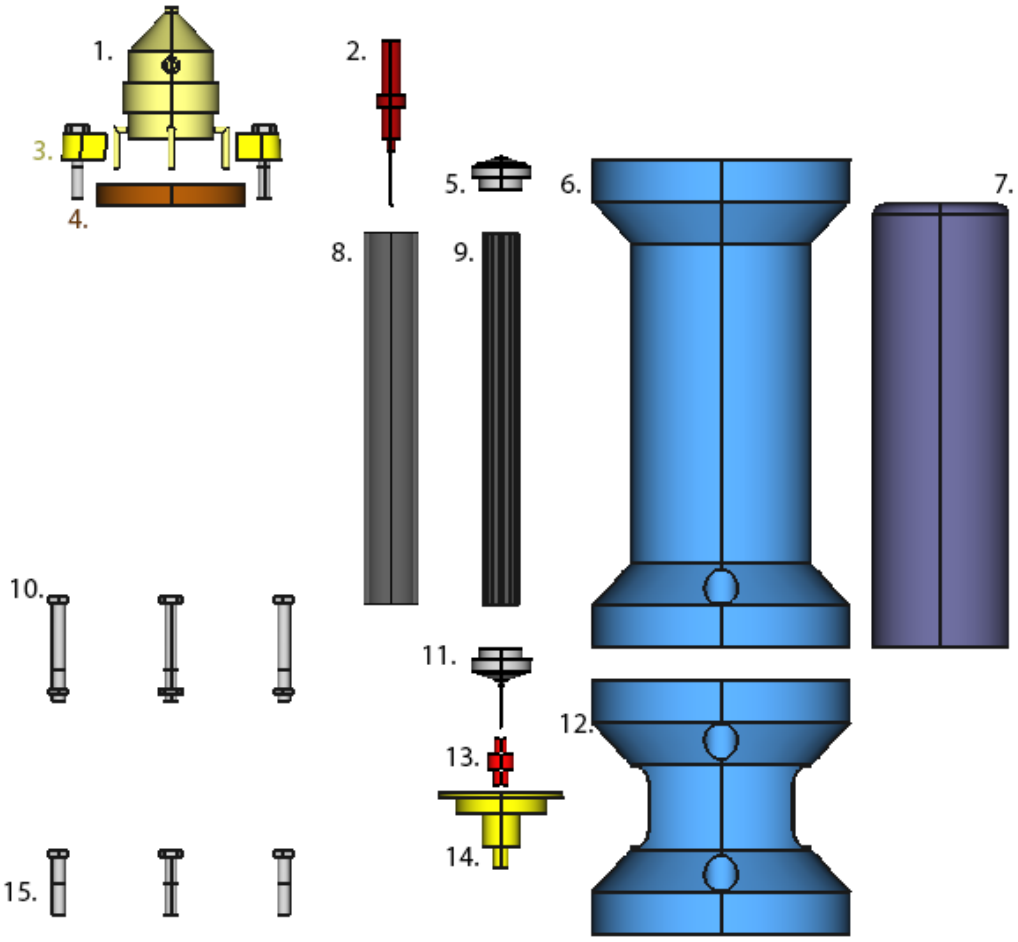


Figure 1.3 Ultracentrifuge First Group Parts Annotations

1. Air Turbine 2.Upper spindle 3.Locks 4.Air Turbine Support 5.Upper Capsule Cap 6.Upper Body 7. Anti-frictional body 8.Capsule 9.Rotor 10.Bolts & Nuts 11.Lower Capsule Cap 12.Lower Body 13. Lower Spindle 14.Lower Spindle Support 15.Bolts

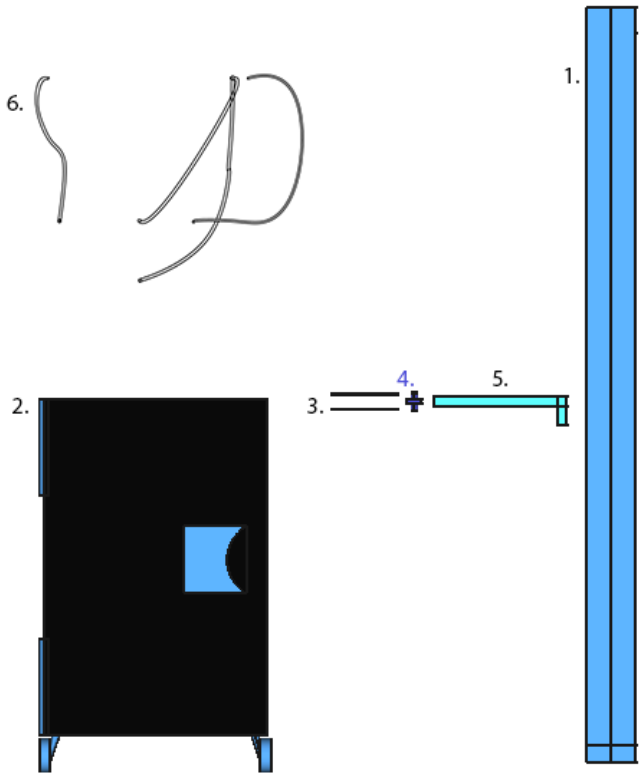
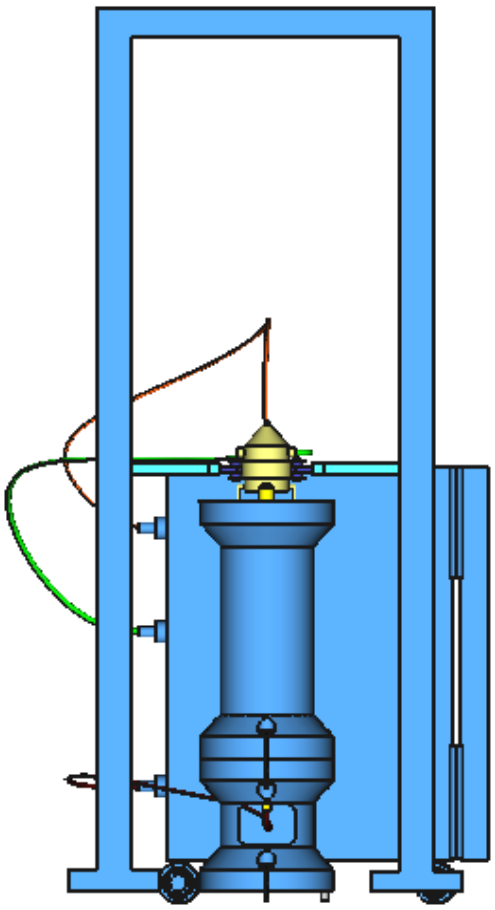
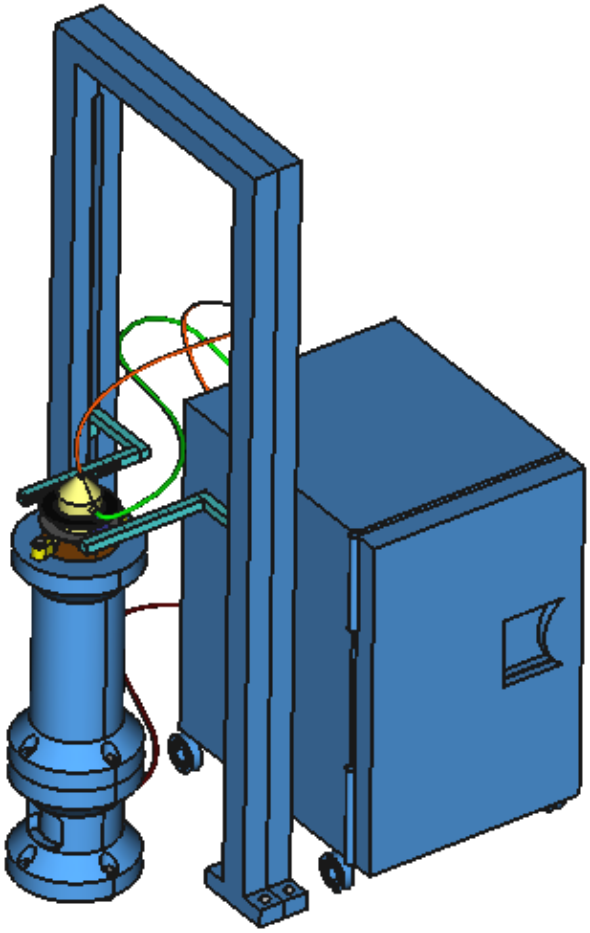


Figure 1.4 ULTRACENTRIFUGE SECOND GROUP PART ANNOTATIONS

1. Lift Support 2.Feeding Machine 3.Air Turbine Lift Holders 4.Horizontal Lift Arm 5.Vertical Lift Arm 6. Tubes

Remark: This Design Is for Educational Purposes. This is just a Prototype Concept Which will Not Work Because Some Parts Are Missing.





15.2 Demonstration and Modelling of the Disc Stack Centrifuge

"To Separate a Liquid from Solid or Liquid from Liquid, We Use the Decanter or the Disc Stack or a Filter Depending On the Amount ofSolids"-Jihad Samarji

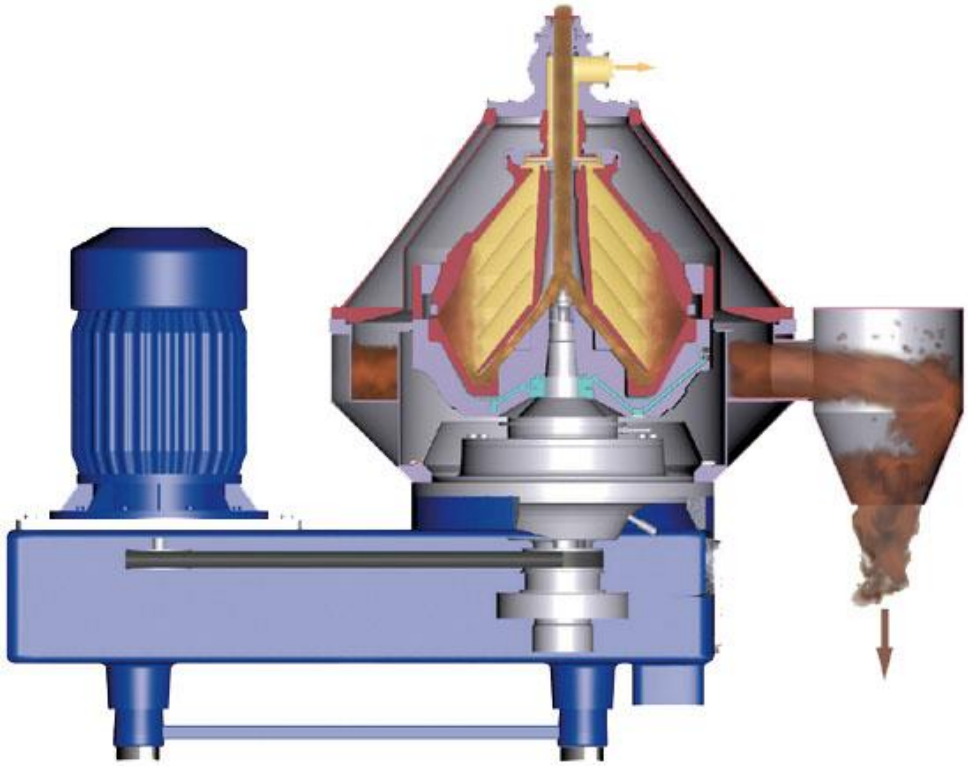


Figure 2.1 Disc Stack Centrifuge

15.2.1 DEVICE DETAILS & SPECIFICATIONS

Conical plate centrifuge (also known as disc bowl centrifuge or disc stack separator) is a type of centrifuge that has a series of conical discs which provides a parallel configuration of centrifugation spaces.

The conical plate centrifuge is used to remove solids (usually impurities) from liquids or to separate two liquid phases from each other by means of an enormously high centrifugal force. The denser solids or liquids which are subjected to these forces move outwards towards the rotating bowl wall while the less dense fluids moves towards the center. The special plates (known as disc stacks) increase the surface settling area which speeds up the separation process. Different stack designs, arrangements and shapes are used for different processes depending on the type of feed present. The concentrated denser solid or liquid is then removed continuously, manually or intermittently, depending on the design of the conical plate centrifuge. This centrifuge is very suitable for clarifying liquids that have small proportion of suspended solids

There is 3 Designs for the disc Stack Centrifuge

In our case, we need to make mass production of substance in big scale and without stopping to clean the disc stack centrifuge from solids so we're going to use the Self-Cleaning Centrifuge.

The Self Cleaning Centrifuge has a Movable plate that operates by the pressure of water underneath it,

When the water is sank, the movable plate goes down to open small nozzles for the solids to pass outside. When all the solids are ejected, water is re-flooded beneath the movable plate to make it goes up and block the nozzles.

We should come up with something similar to this:

Utilities consumption	
Electric power	max. 46 kW
Operating liquid during discharge	10 l/h
Cooling water, jacket	300 l/h
Cooling water, oil	80 l/h
Sealing liquid	100 l/h
Flushing liquid, per discharge	25–30 l
ATEX design codes	
BD 95X: EX II 2 G T4 X for zone 1 & 2	Inert gas design
BD 95Y: EX II 3 G T4 X for zone 2	Electrically protected
Material data	
Bowl body, hood and lock ring	s.s. 1.4418
Frame top part and hood	s.s. 1.4401 UNS 31600
Frame bottom part	Cast iron
Gaskets and O-rings	Fluorocarbon rubber

Technical specifications	
Throughput capacity	max. 52 m³/h
Bowl speed	4,300 rpm
Bowl volume	66 l
Sludge space	17 l
Motor speed synchron. 60.7 Hz	1,821 rpm
Motor power installed	52 kW
Starting time	6–8 min
Stopping time without brake	80 min
Inlet pressure at 46 m³/h	300 kPa
Outlet pressure, methyl ester phase	min. 200 kPa
Outlet pressure, heavy phase	800 kPa
Sound pressure	78 dB(A)
Overhead hoist lifting capacity	min. 1,200 kg

Table2.1 Disc Stack Centrifuge Specifications. See also figure 2.1 and 2.2

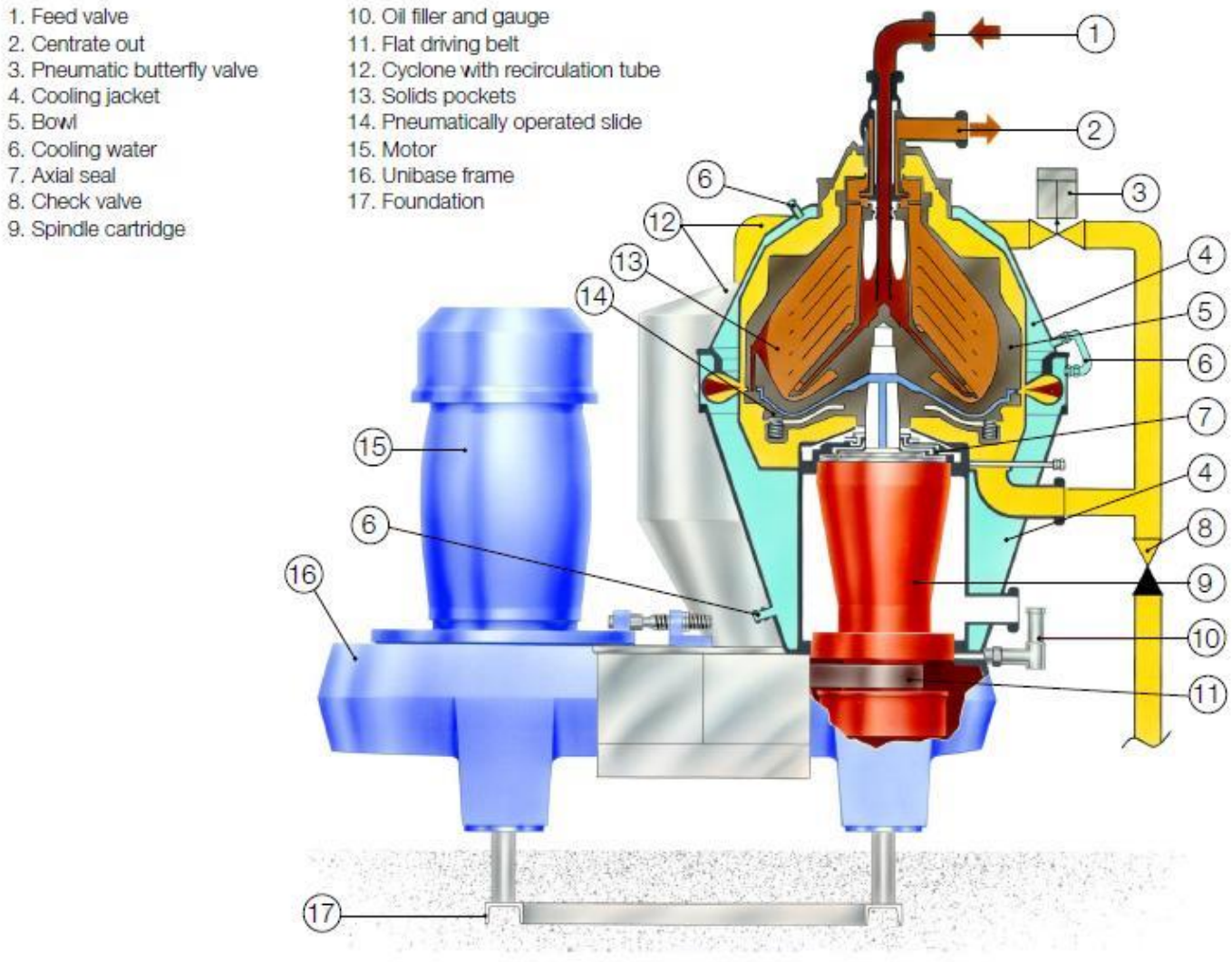
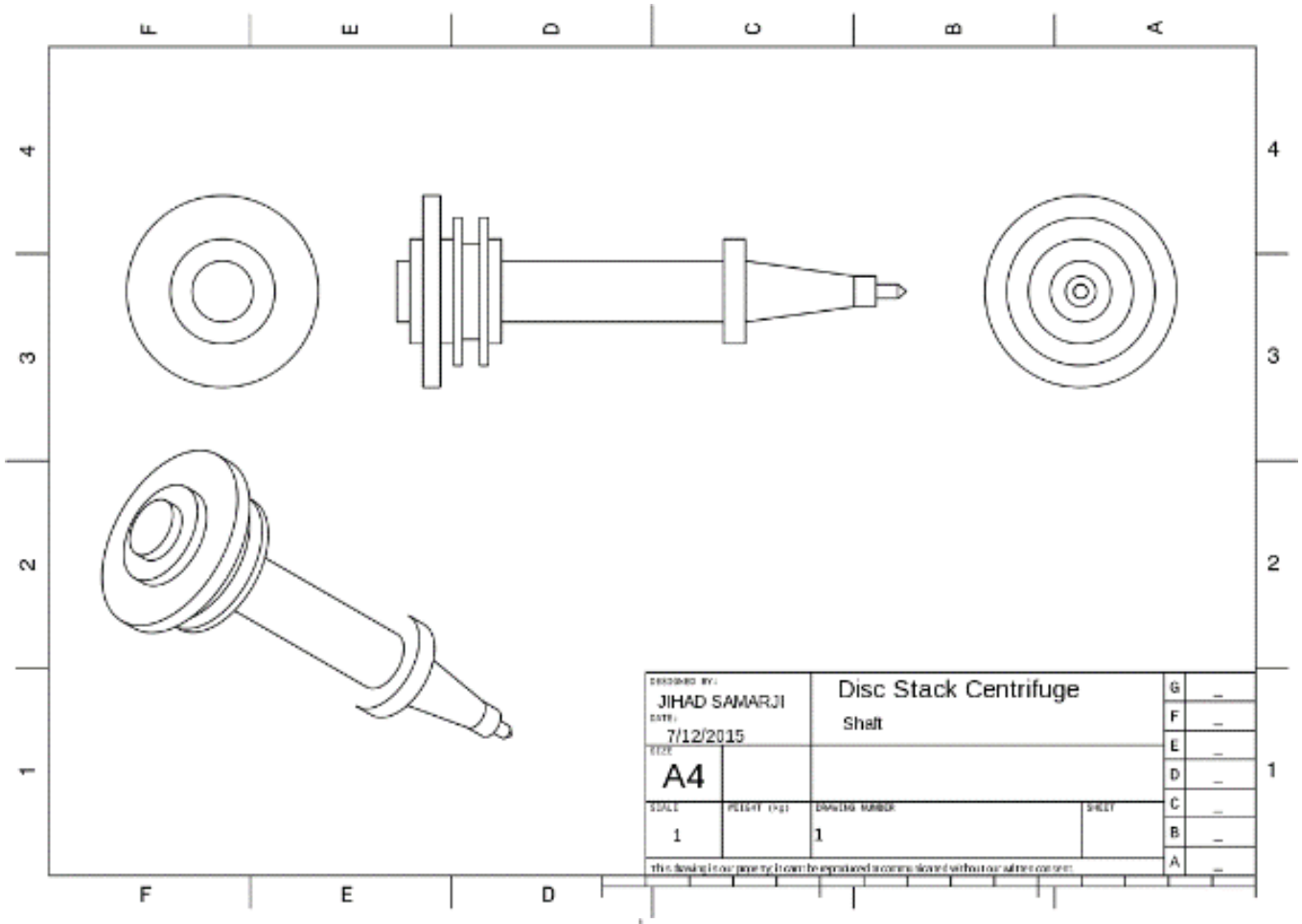
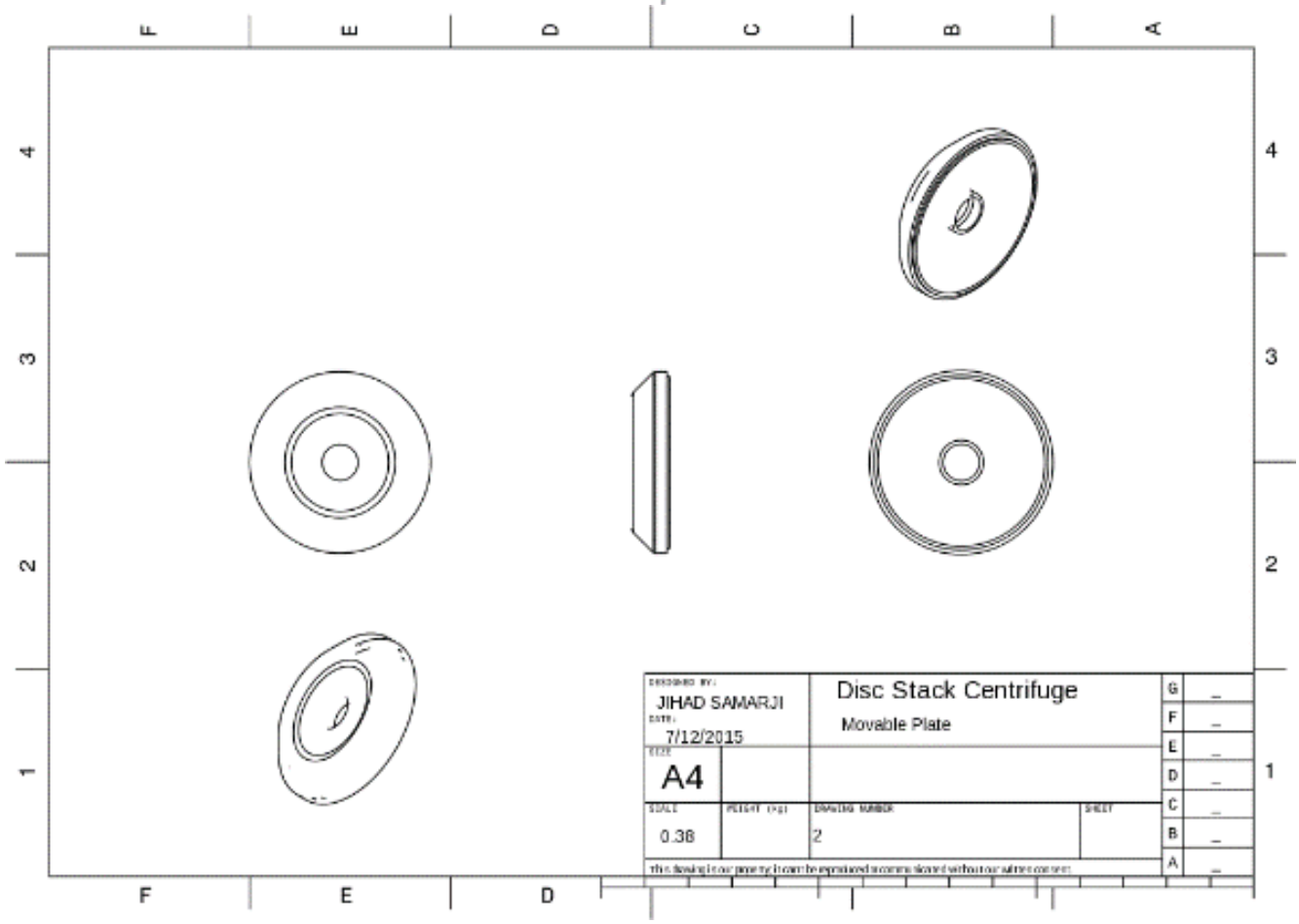
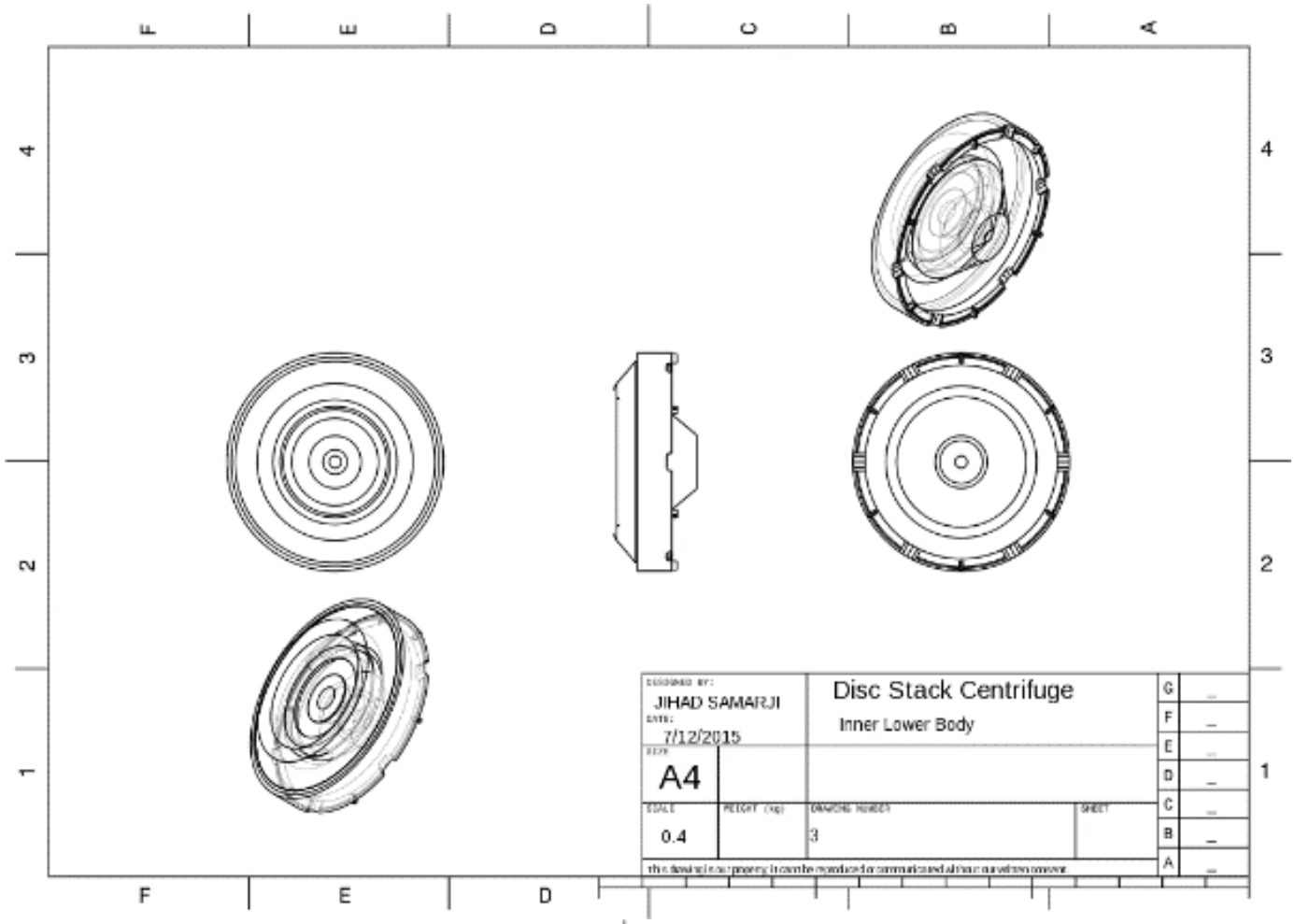


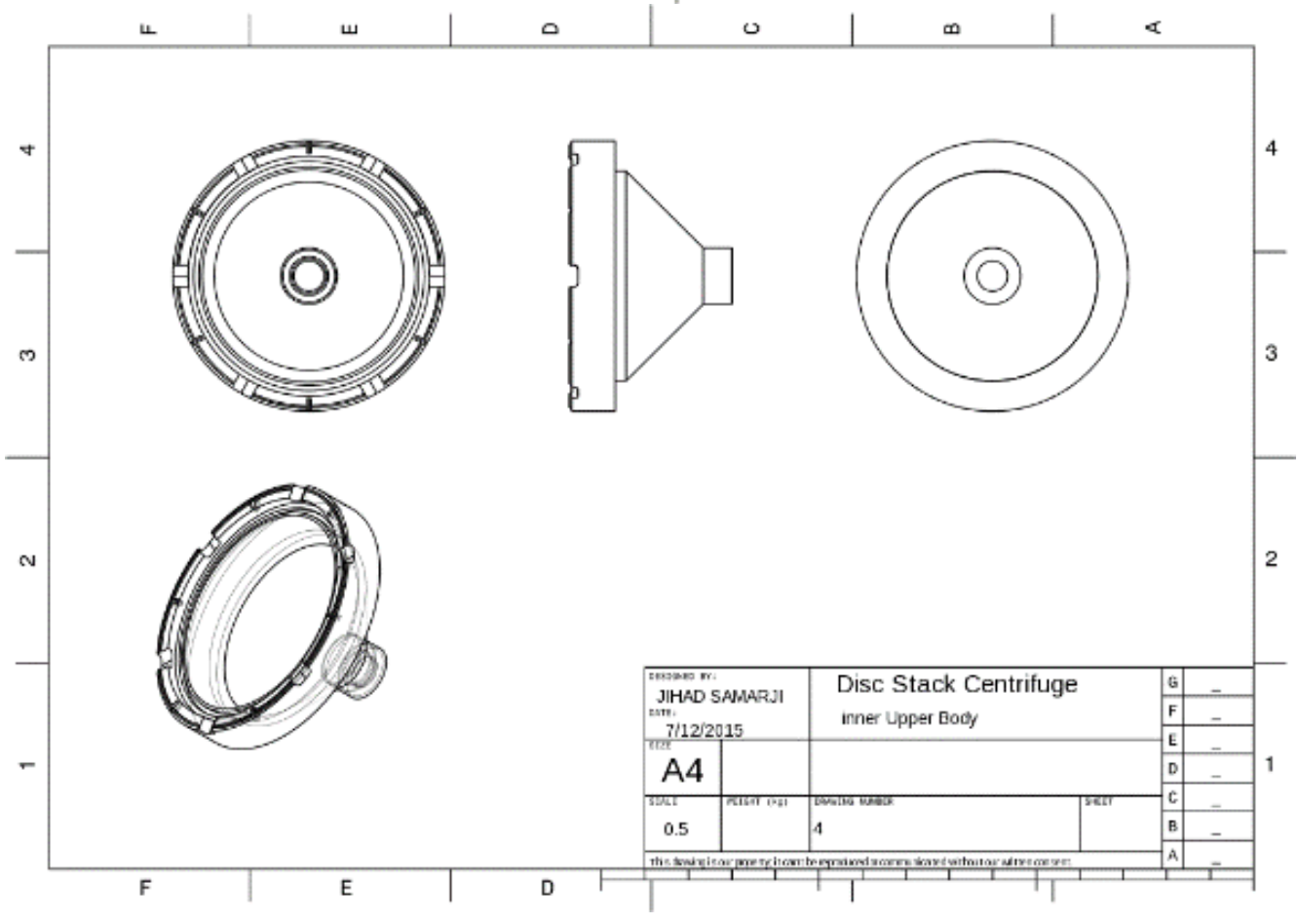
Figure 2.21 Disc Stack Centrifuge Annotation

15.2.2 PART DIMENSIONS & DESIGN


JIHAD SAMARJI
7/12/2015
A4
1
1
Disc Stack Centrifuge
Shaft


JIHAD SAMARJI
7/12/2015
A4
0.38
2
Disc Stack Centrifuge
Movable Plate


DESIGNED BY:
JIHAD SAMARJI
DATE:
7/12/2015
SIZE
A4
SCALE
0.4
WEIGHT (kg)
DRAWING NUMBER
3
SHEET
Disc Stack Centrifuge
Inner Lower Body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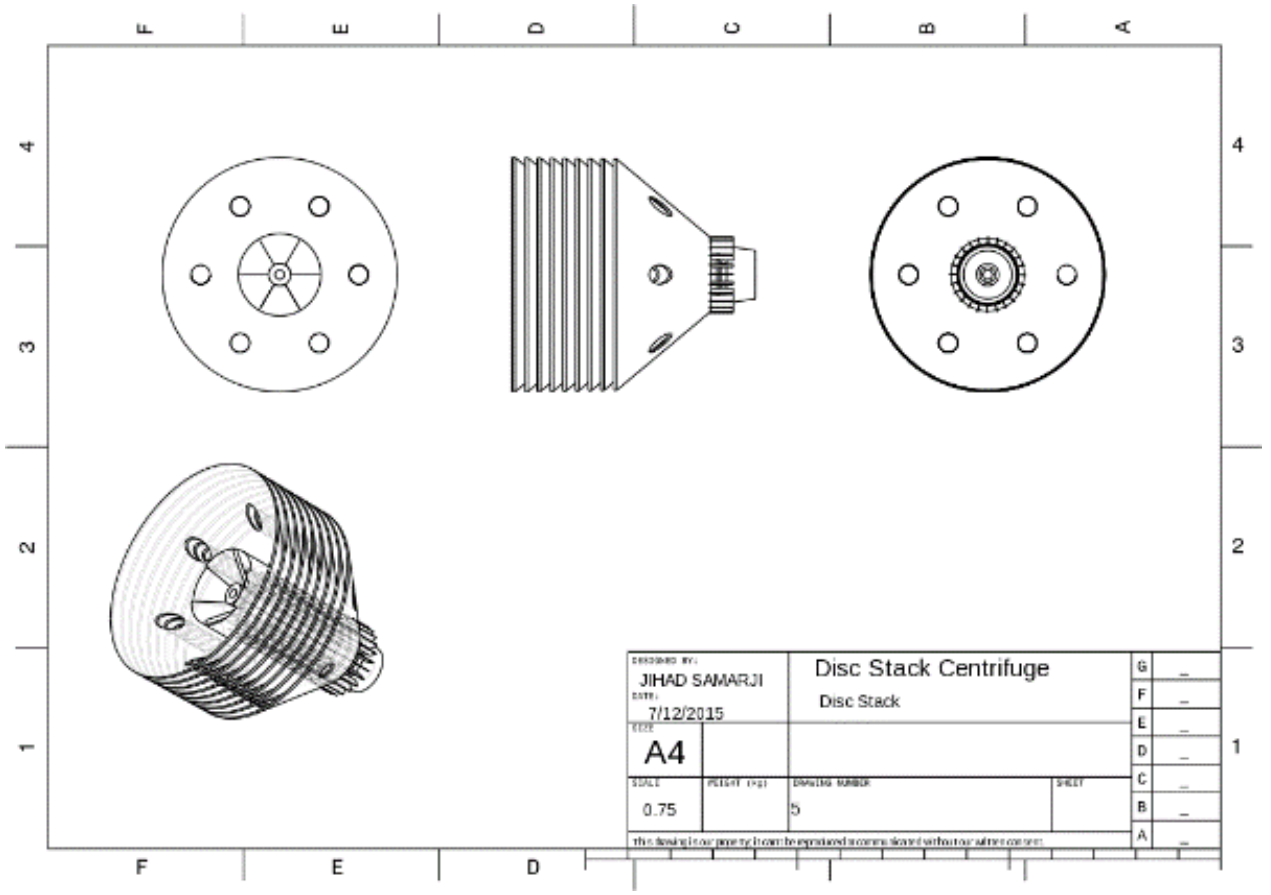
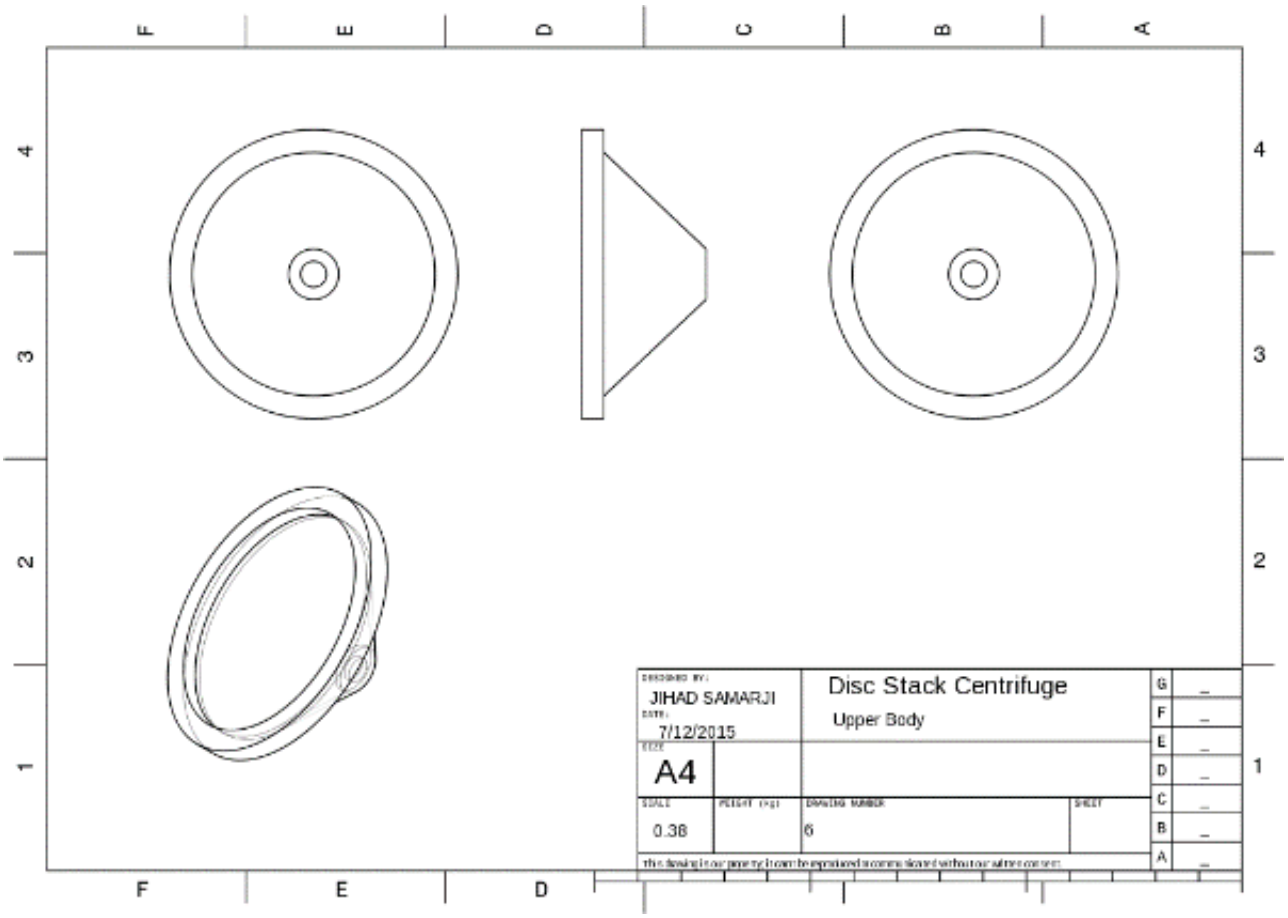
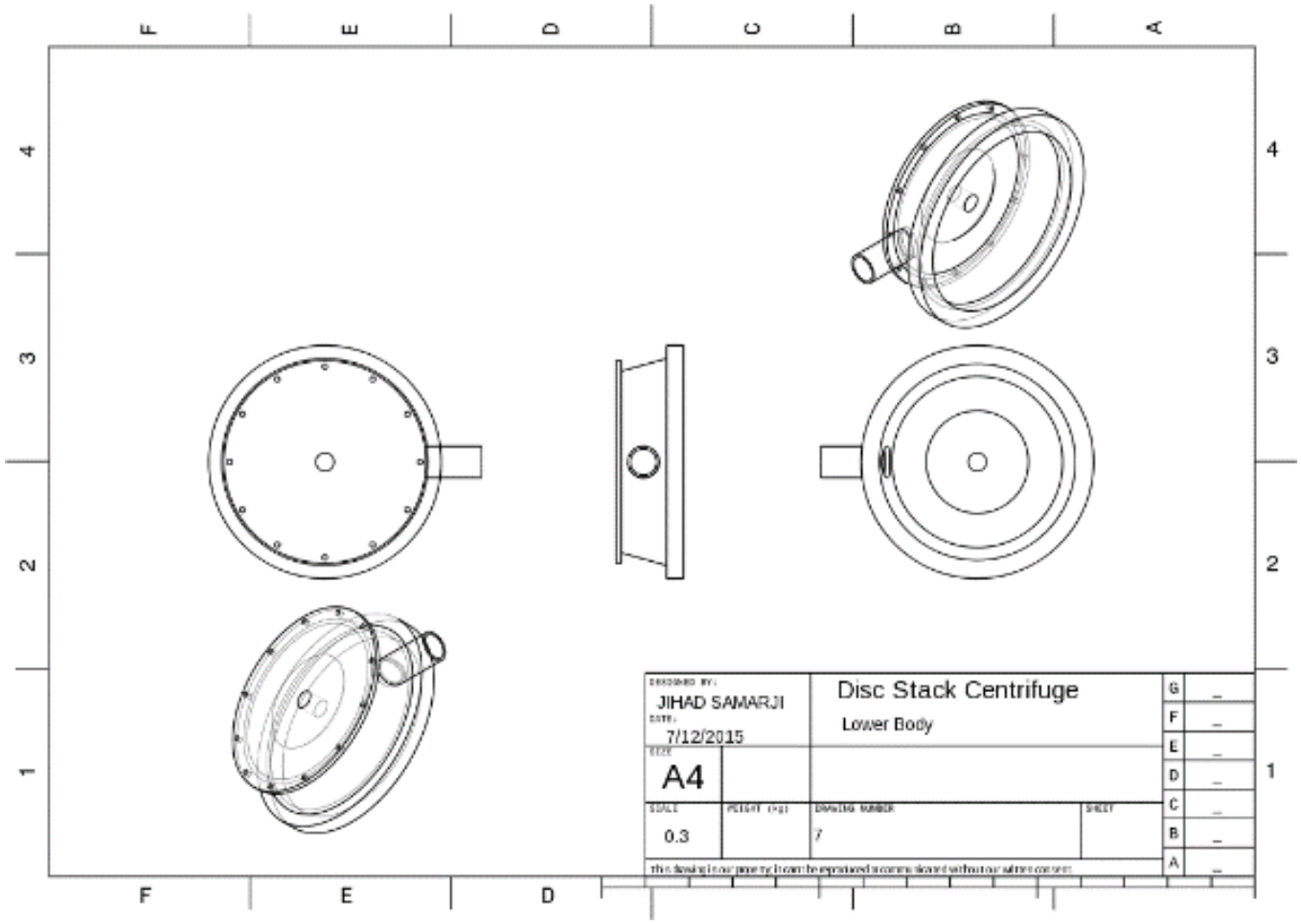
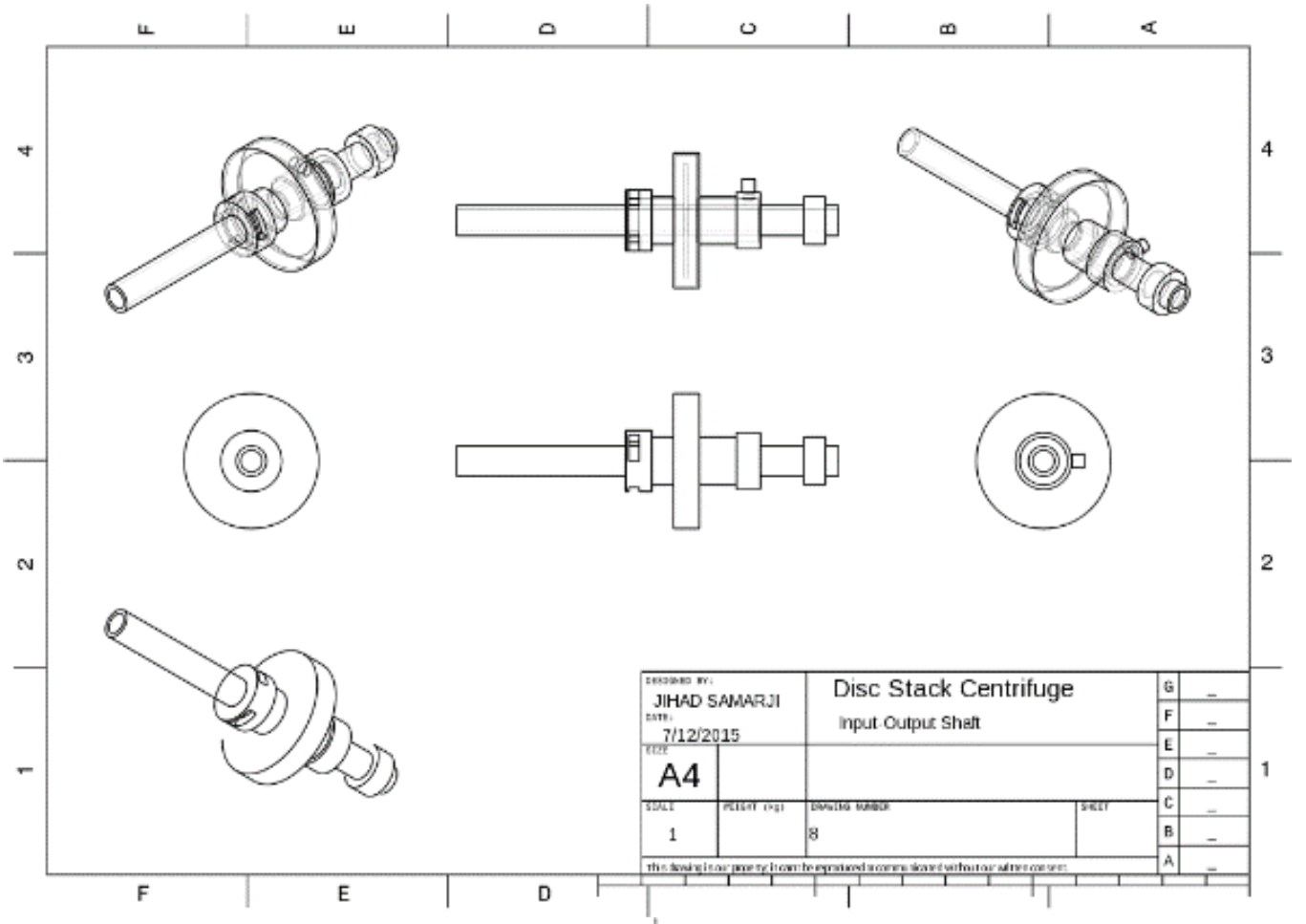
JIHAD SAMARJI
DATE:
7/12/2015
SIZE
A4
SCALE
0.5
WEIGHT (kg)
DRAWING NUMBER
4
SHEET
Disc Stack Centrifuge
inner Upper Body

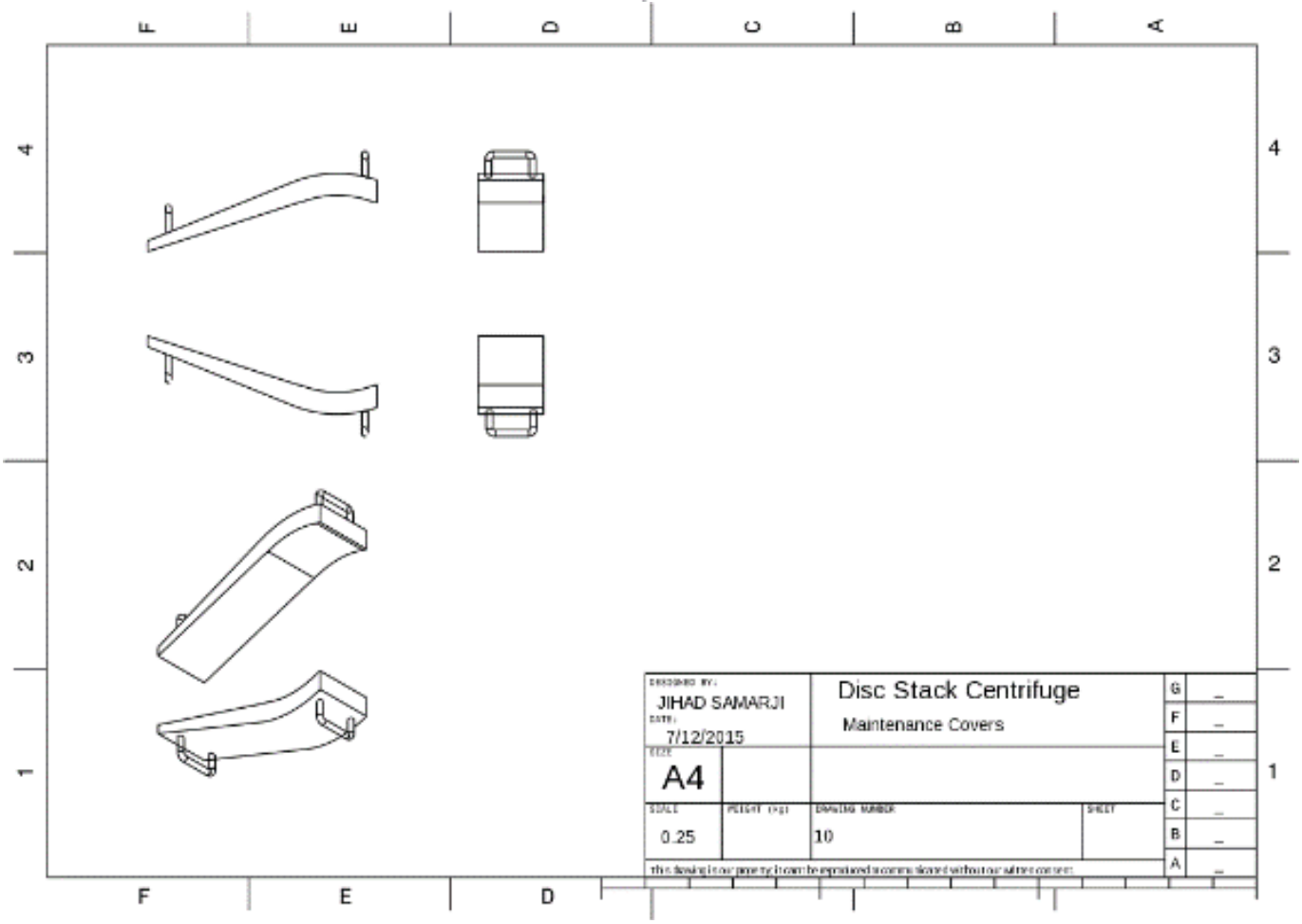

JIHAD SAMARJI
7/12/2015
A4
0.75
Disc Stack Centrifuge
Disc Stack
5


JIHAD SAMARJI
7/12/2015
A4
0.38
Disc Stack Centrifuge
Upper Body
6

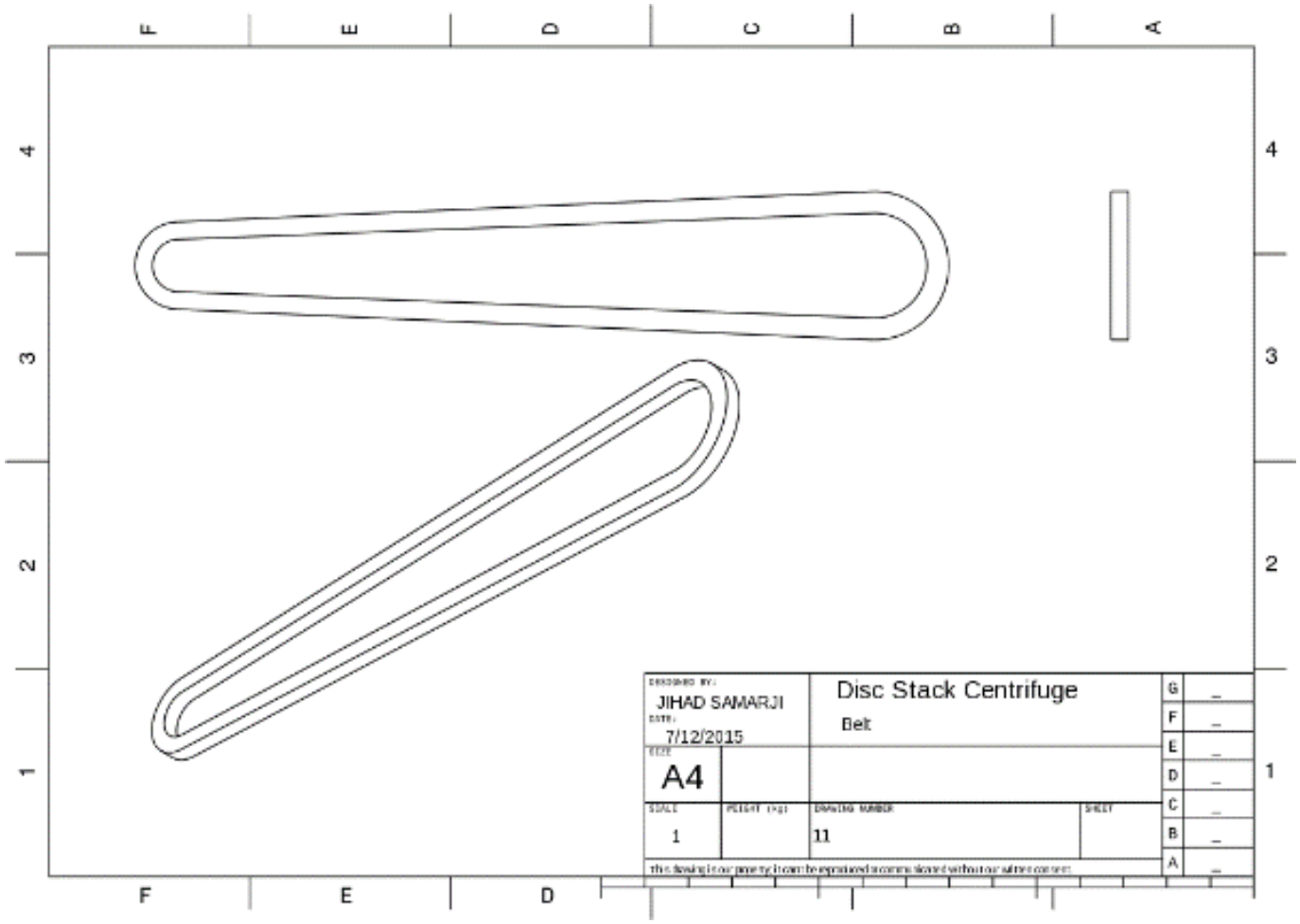

F
E
D
C
B
A
4
3
2
1
DESIGNED BY:
JIHAD SAMARJI
DATE:
7/12/2015
SIZE
A4
SCALE
0.3
WEIGHT (kg)
DRAWING NUMBER
SHEET
Disc Stack Centrifuge
Lower Body
G
F
E
D
C
B
A

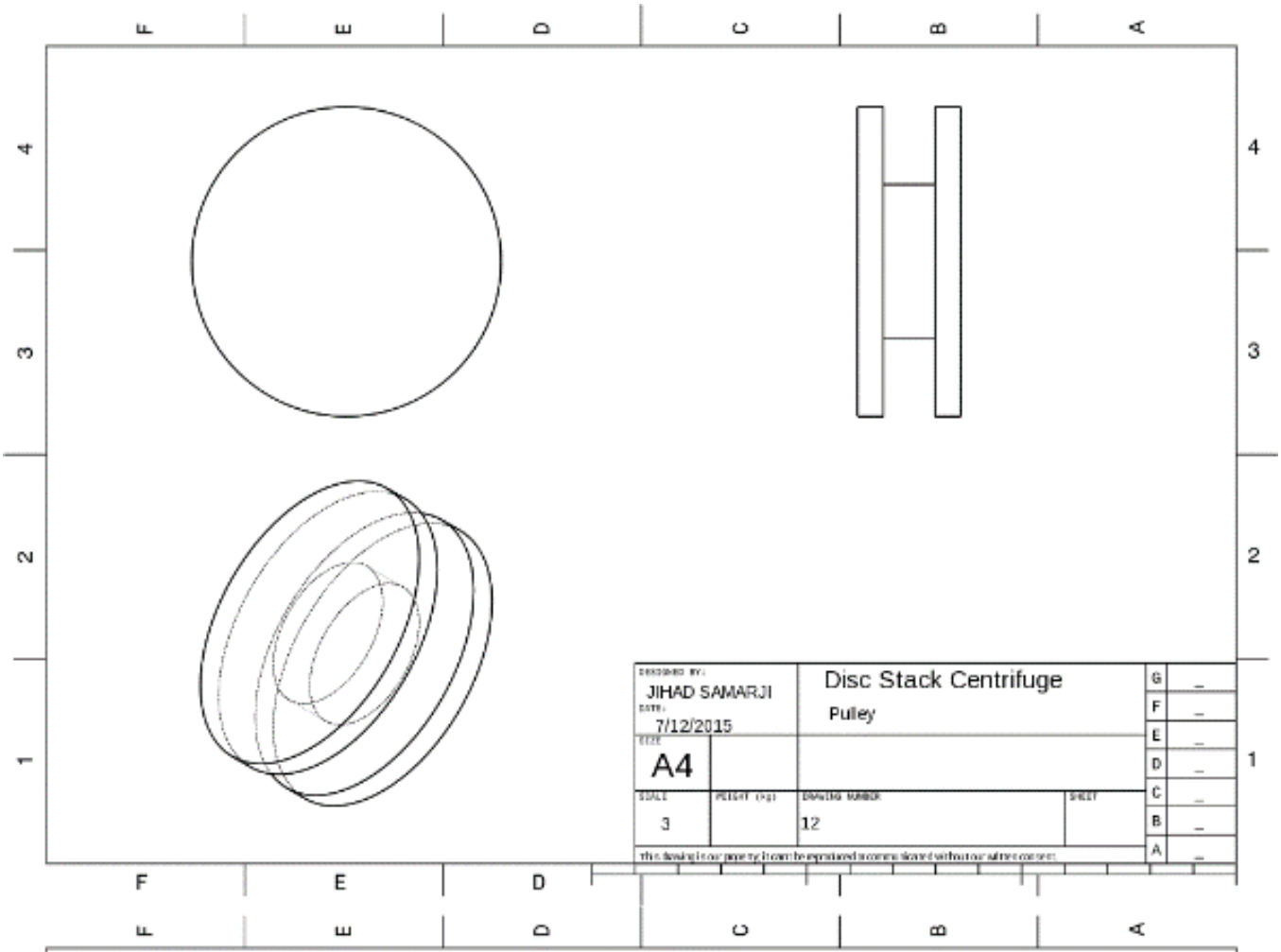


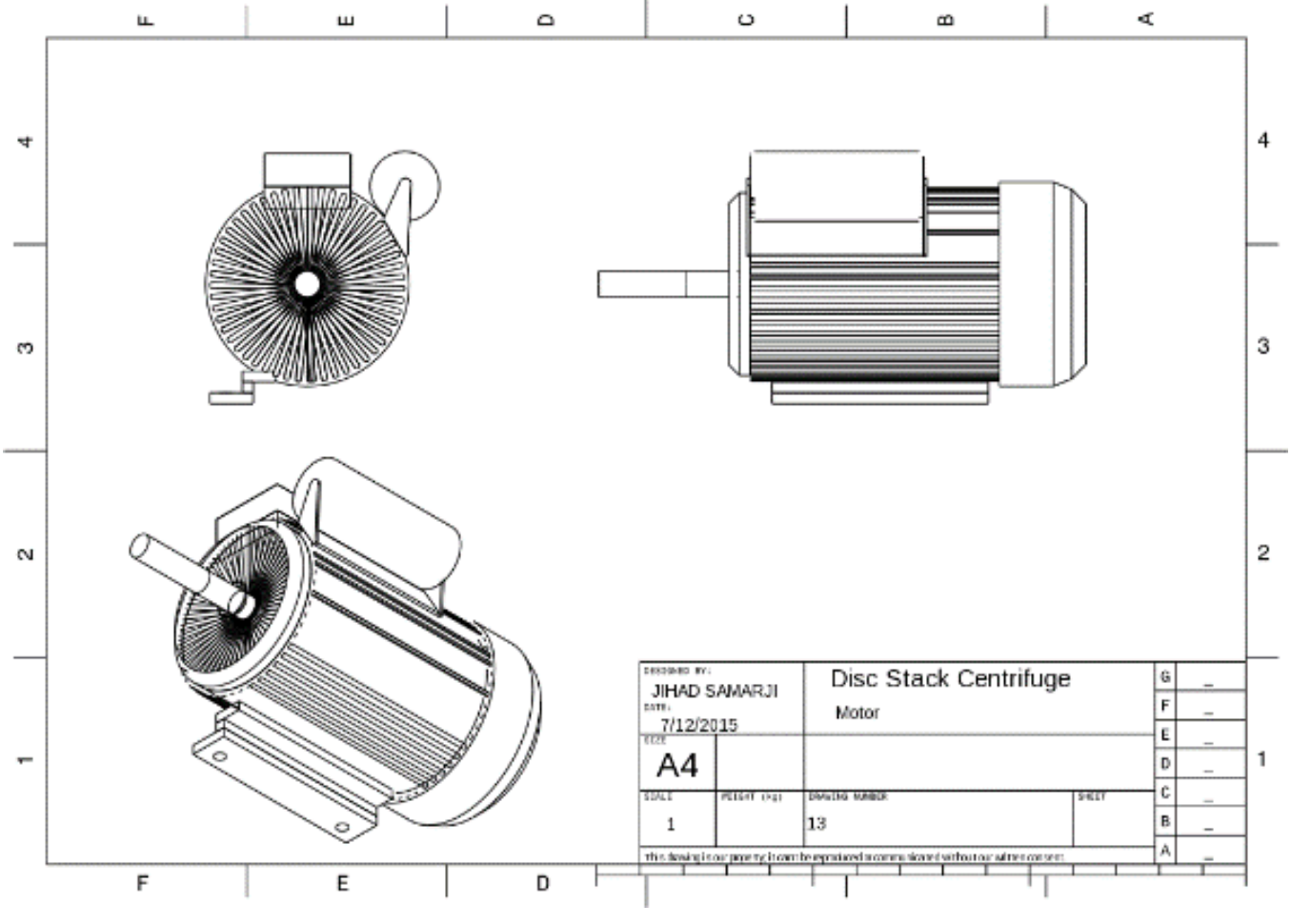
DESIGNED BY: JIHAD SAMARJI	Disc Stack Centrifuge		G _
DATE: 7/12/2015	Input Output Shaft		F _
SIZE A4			E _
			D _
SCALE 1	WEIGHT (kg)	DRAWING NUMBER 8	SHEET	C _
this drawing is our property it can't be reproduced in any kind without our written consent			B _
			A _



DESIGNED BY: JIHAD SAMARJI	Disc Stack Centrifuge		G _
DATE: 7/12/2015	Maintenance Covers		F _
SIZE A4			E _
			D _
SCALE 0.25	WEIGHT (kg)	DRAWING NUMBER 10	SHEET	C _
this drawing is our property it can't be reproduced in any kind without our written consent			B _
			A _


F
E
D
C
B
A
4
3
2
1
DESIGNED BY:
JIHAD SAMARJI
DATE:
7/12/2015
SIZE
A4
SCALE
1
WEIGHT (kg)
DRAWING NUMBER
11
SHEET
Disc Stack Centrifuge
Belt
G
F
E
D
C
B
A


JIHAD SAMARJI
7/12/2015
A4
Disc Stack Centrifuge
Pulley
3
12


JIHAD SAMARJI
7/12/2015
A4
Disc Stack Centrifuge
Motor
1
13

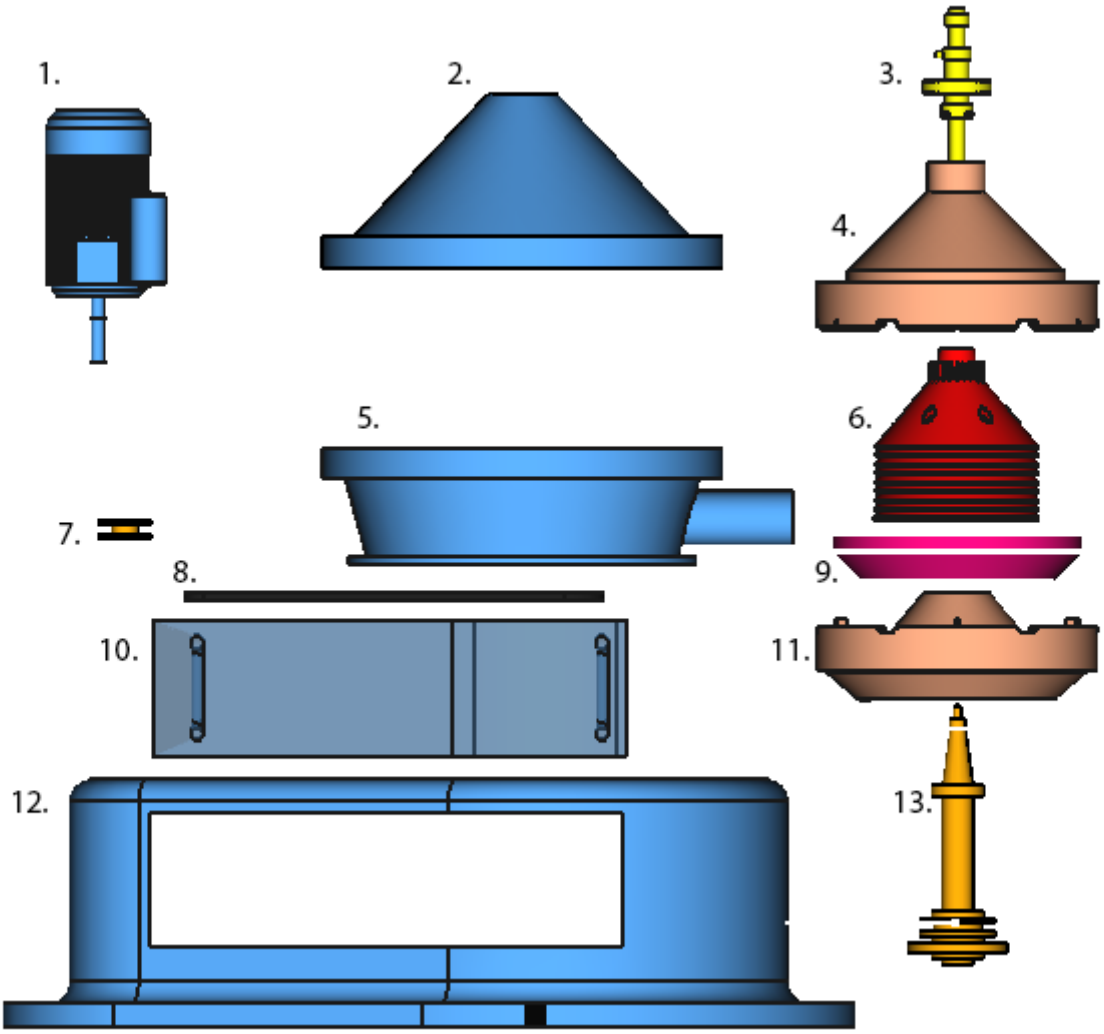
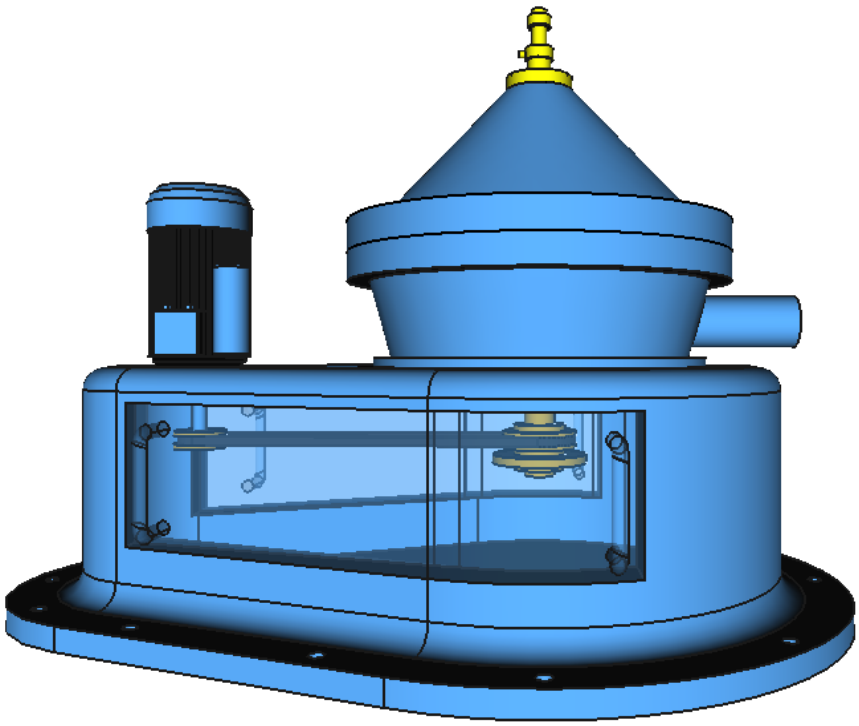


Figure 2.3 Disc Stack Centrifuge Annotations

1. Motor 2.Upper Body 3.Inlet-Outlet Shaft 4.Inner Upper Body 5.Lower Body 6.Disc Stack 7. Pulley 8.Belt 9.Movable Plate 10.Maintenance Cover 11.Inner Lower Body 12.Support 13. Shaft



15.3 Chapter 3: Demonstration and Modelling of the Ultrafiltration.

"To Filtrate a Huge Supply of Substance or Water we need an Ultrafiltration Device"-Jihad Samarji

Figure 3.1 Ultrafiltration Machine

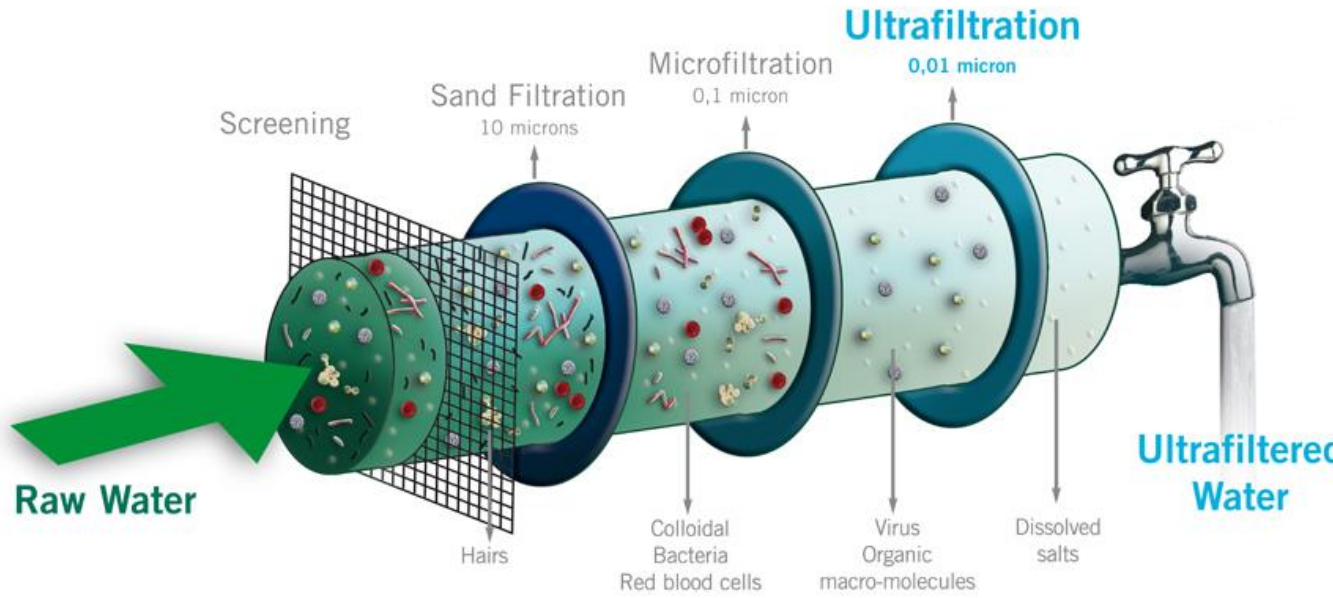


Figure 3.2 a small demonstration about the Ultrafiltration Process

15.3.1 DEVICE DETAILS & SPECIFICATIONS

Ultrafiltration (UF) is a variety of membrane filtration in which forces like pressure or concentration gradients lead to a separation through a membrane. Suspended and solutes of high molecular weight are retained in the so-called retentive, while water and low molecular weight solutes pass through the membrane in thepermeate. This separation process is used in industry and research for purifying and concentrating macromolecular (103 - 106 Da) solutions, especially protein solutions. Ultrafiltration is not fundamentally different from microfiltration. Both of these separate based on size exclusion or particle capture. It is fundamentally different from membrane gas separation, which separate based on different amounts of absorption and different rates of diffusion. Ultrafiltration membranes are defined by the molecular weight cut-off(MWCO) of the membrane used. Ultrafiltration is applied in cross-flow or dead-end mode.

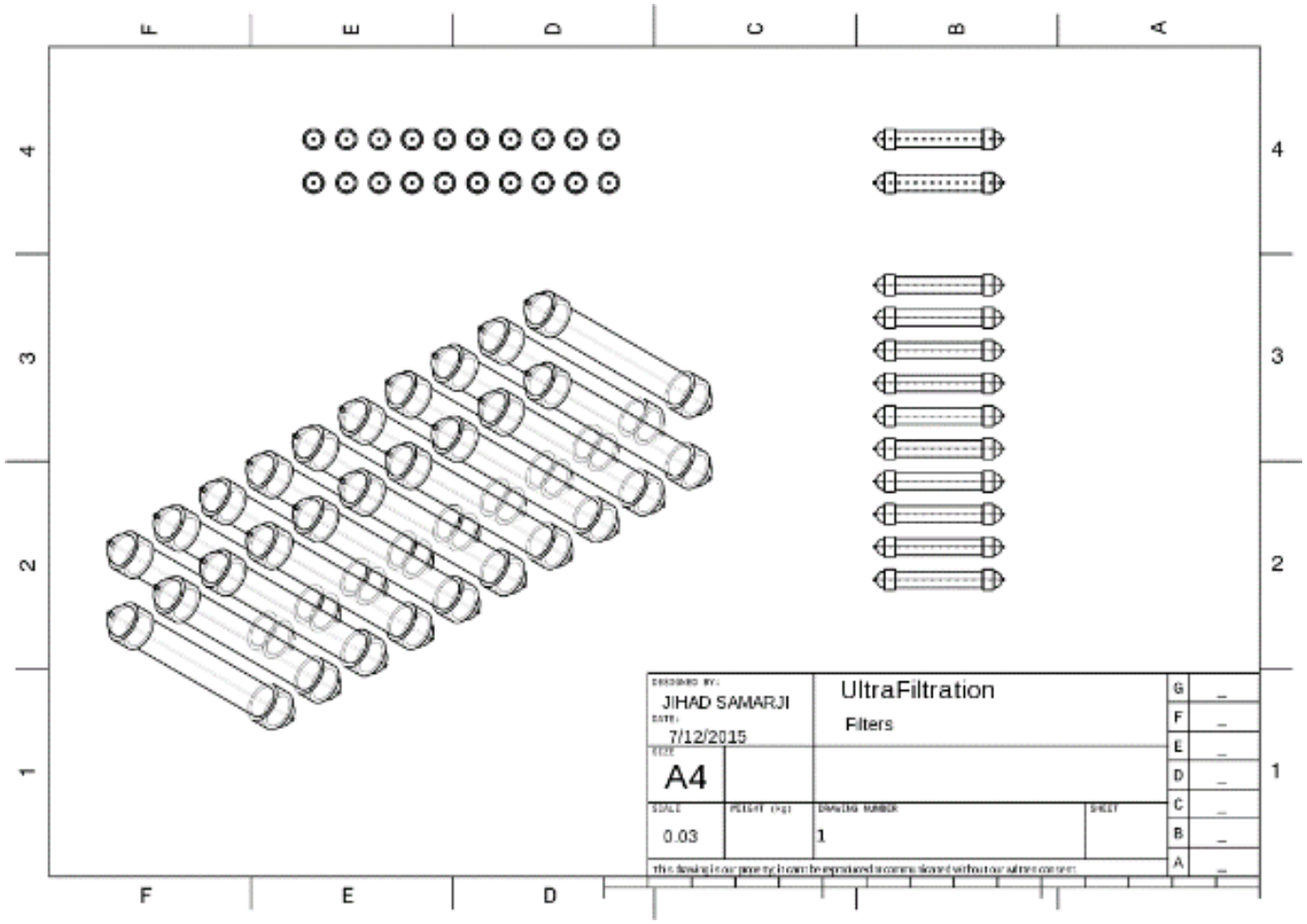
Ultrafiltration is used in Industries such as chemical and pharmaceutical manufacturing, food and beverage processing, and waste water treatment, employ ultrafiltration in order to recycle flow or add value to later products. Blood dialysis also utilizes ultrafiltration.

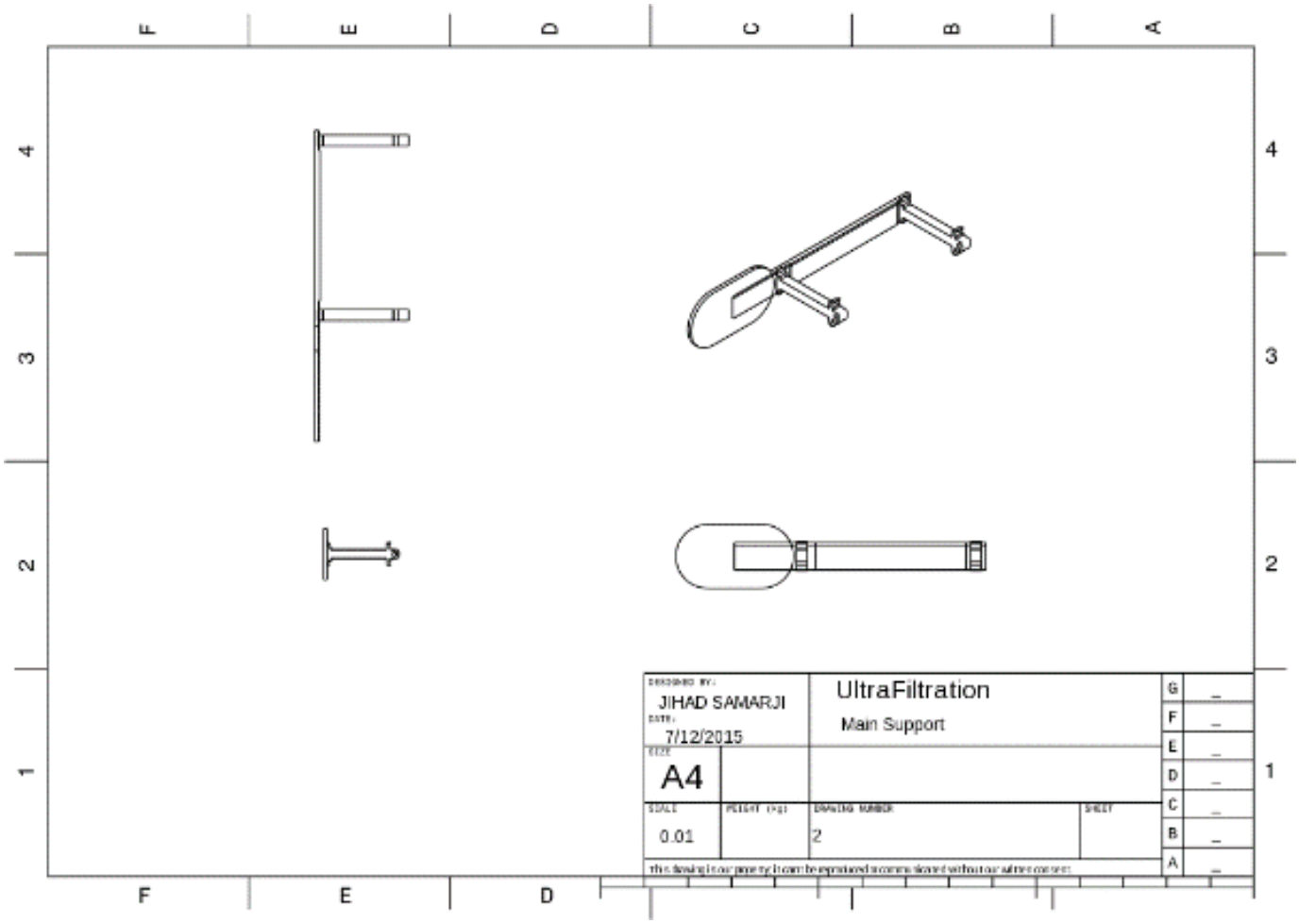
In our case we're going to use the ultrafiltration device for Protein concentration. See Figure.3.1 and 3.2

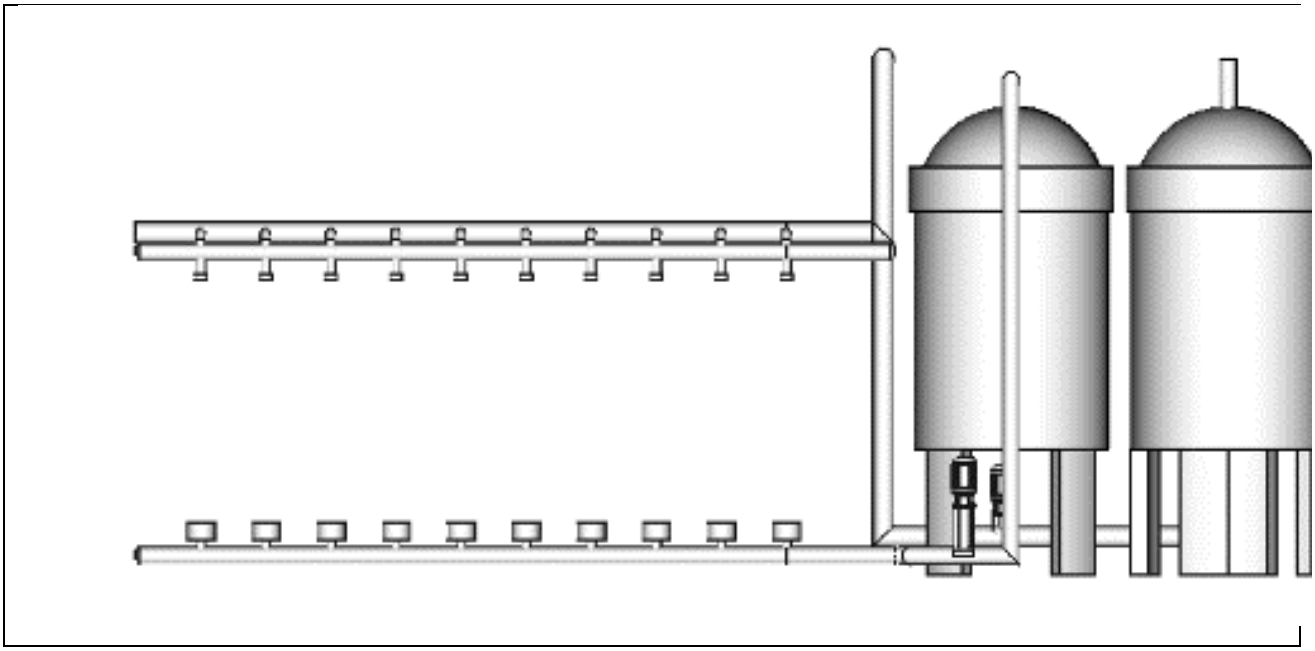
Foulant	Reagent	Time and Temperature	Mode of Action
Fats and oils, proteins, polysaccharides, bacteria	0.5M NaOH with 200 ppm Cl2	30-60 min 25-55 °C	Hydrolysis and oxidation
DNA, mineral salts	0.1M – 0.5M acid (acetic, citric, nitric)	30-60 min 25-35 °C	Solubilization
Fats, oils, biopolymers, proteins	0.1% SDS, 0.1% Triton X-100	30 min – overnight 25-55 °C	Wetting, emulsifying, suspending, dispersing
Cell fragments, fats, oils, proteins	Enzyme detergents	30 min – overnight 30 – 40 °C	Catalytic breakdown
DNA	0.5% DNAase	30 min – overnight 20 – 40 °C	Enzyme hydrolysis

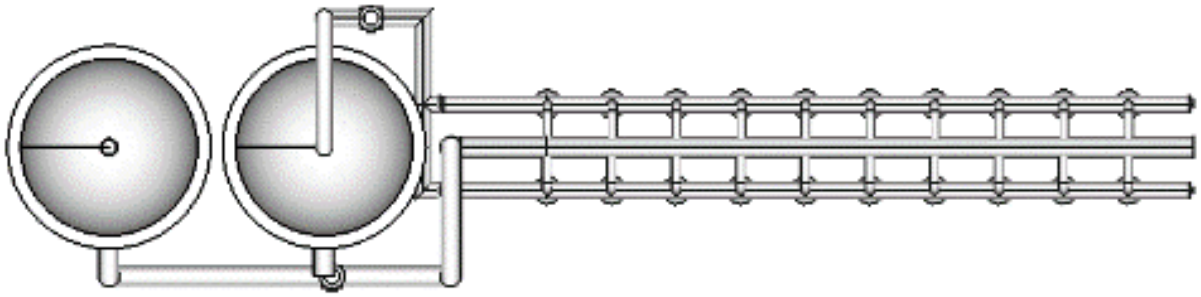
Table 3.2 SUMMARY OF COMMON TYPES OF FOULING AND THEIR RESPECTIVE CHEMICAL TREATMENTS

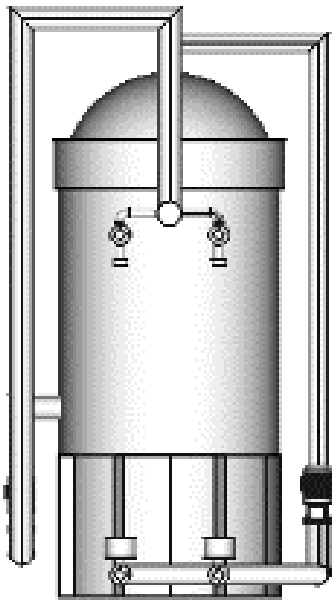
15.3.2 PART DIMENSIONS & DESIGN

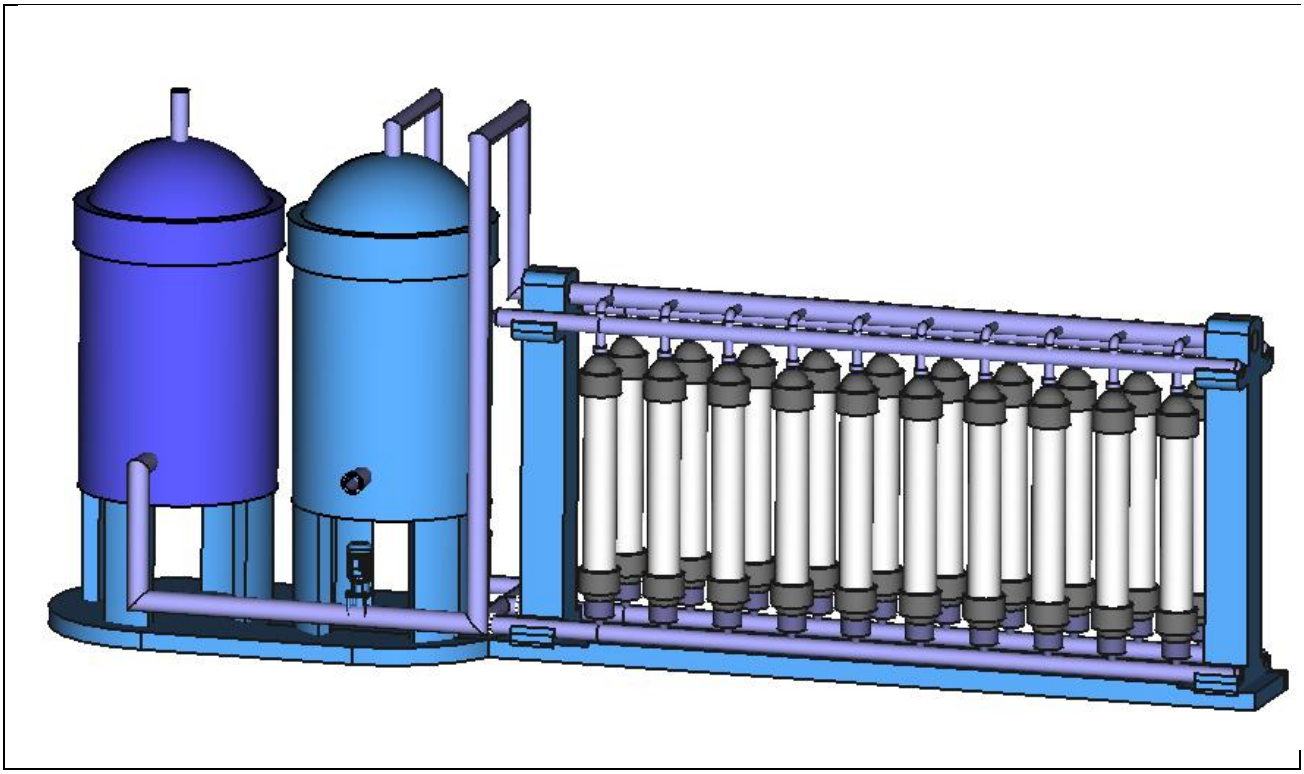

F
E
D
C
B
A
4
3
2
1
DESIGNED BY:
JIHAD SAMARJI
DATE:
7/12/2015
SIZE
A4
SCALE
0.03
WEIGHT (kg)
DRAWING NUMBER
1
SHEET
UltraFiltration
Filters
G
F
E
D
C
B
A


F
E
D
C
B
A
4
3
2
1
DESIGNED BY:
JIHAD SAMARJI
DATE:
7/12/2015
SIZE
A4
SCALE
0.01
WEIGHT (kg)
DRAWING NUMBER
2
SHEET
UltraFiltration
Main Support
this drawing is our property it can be reproduced or considered without our written consent
G
F
E
D
C
B
A









The Ultrafiltration is Simple, no need for further dimensions nor annotations. It contains 2 Tanks, one for filtrated substance and the other for non-filtrated substance which is connected to a set of filters. The device also contains 2 pumps to boost the substance into the pipes. For more information about the ultrafiltration process, see figure.5

15.4 Demonstration and Modelling of the Chromatography Device

-ZaherChendeb-

15.4.1 DEVICE DETAILS & SPECIFICATIONS

AxiChrom<Chromatography>

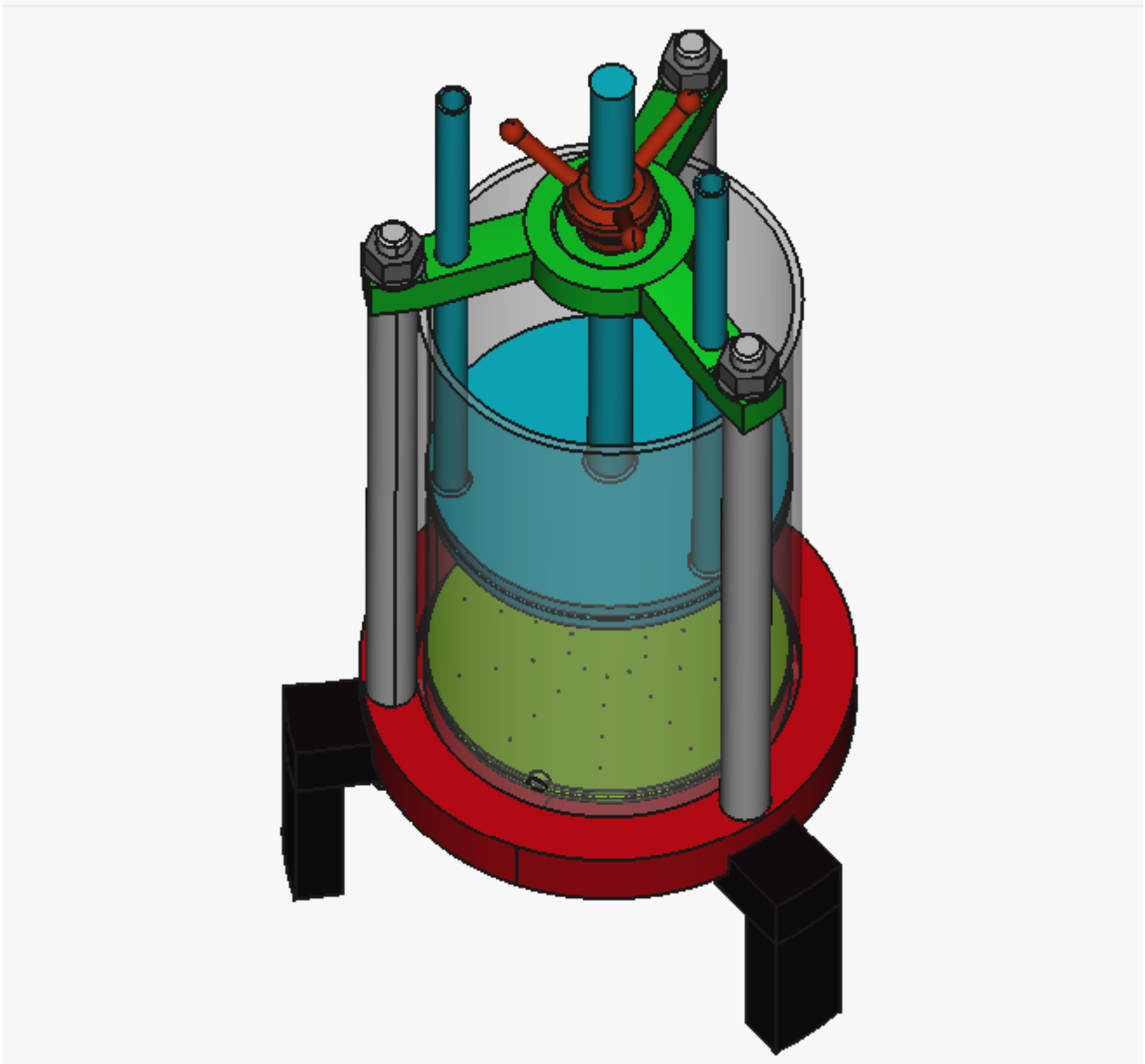
The AxiChrom column platform is a revolutionary concept in column chromatography that simplifies column handling at all scales from process development to full-scale production. AxiChrom columns introduce three key features – Intelligent Packing, Intuitive handling, and Predictable scale-up – that together make process chromatography easier, safer, and more efficient.

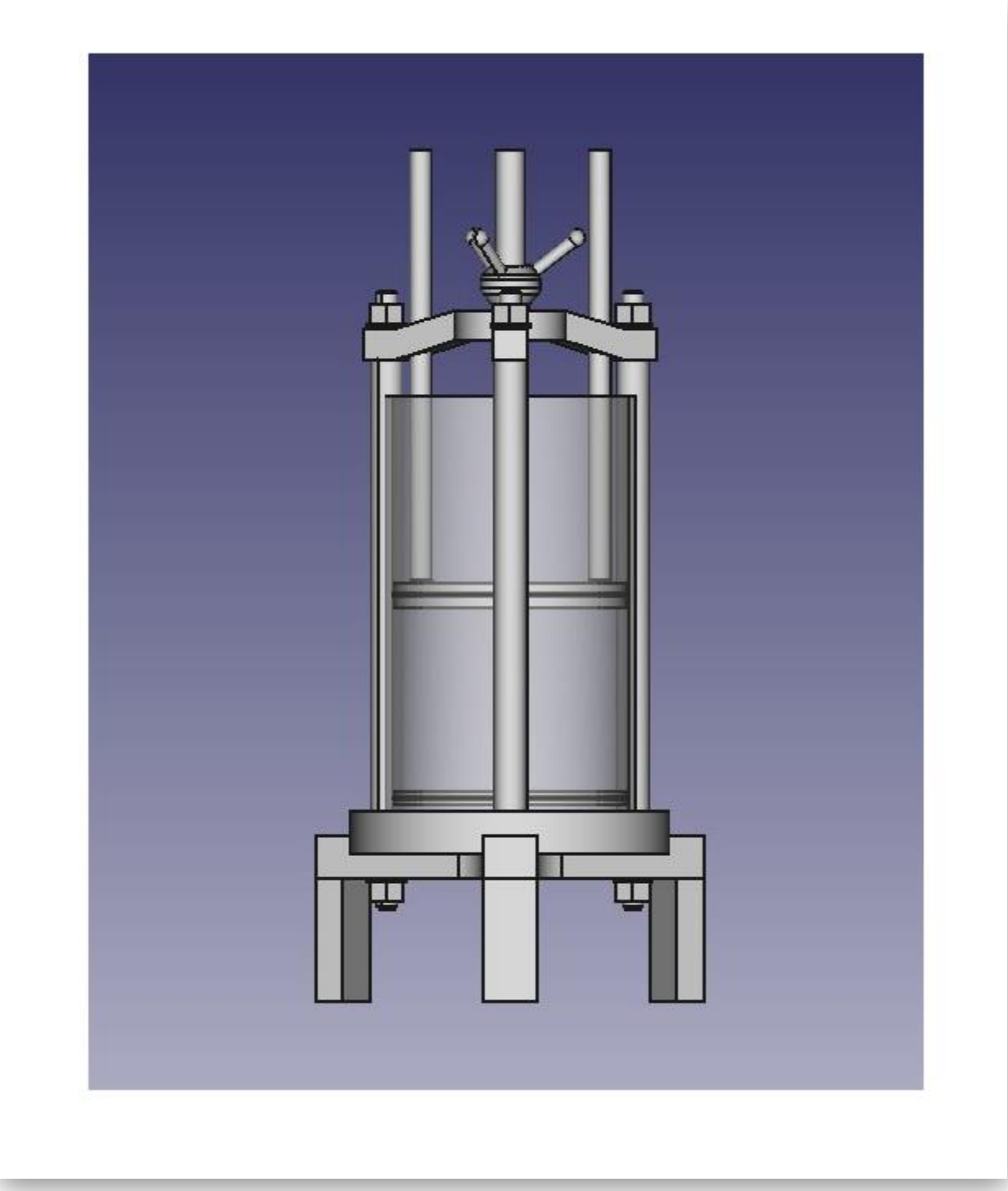
The AxiChrom process column family has been designed to deliver reproducible results from process development to production scales. This is facilitated by the innovative Intelligent Packing where UNICORN™ software, ÄKTA™ systems and AxiChrom columns work together to facilitate a convenient operation for packing of the bed via axial compression.

The Intended use of The AxiChrom family of process columns has been designed for low pressure chromatographic separation of biomolecules such as proteins, peptides and oligonucleotides in GMP-regulated environments.

The AxiChrom columns are intended for production use only and should not be used for diagnostic purposes in any clinical or in vitro procedures. The columns are not suitable for operation in a potentially explosive atmosphere or for handling flammable liquids. If the columns are used for purposes other than those specified in the user documentation, safe operation and the protection provided by the system may be impaired.

15.4.2 PART DIMENSIONS & DESIGN

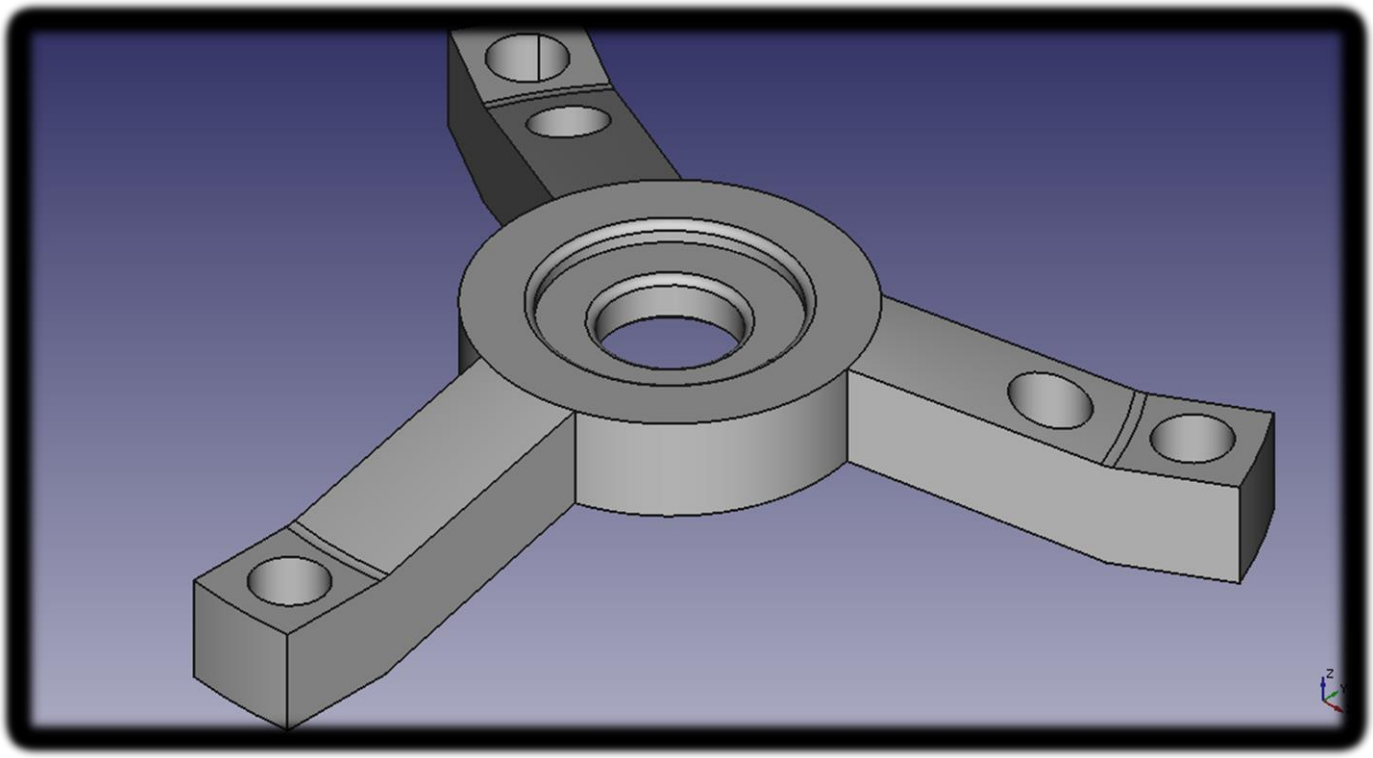


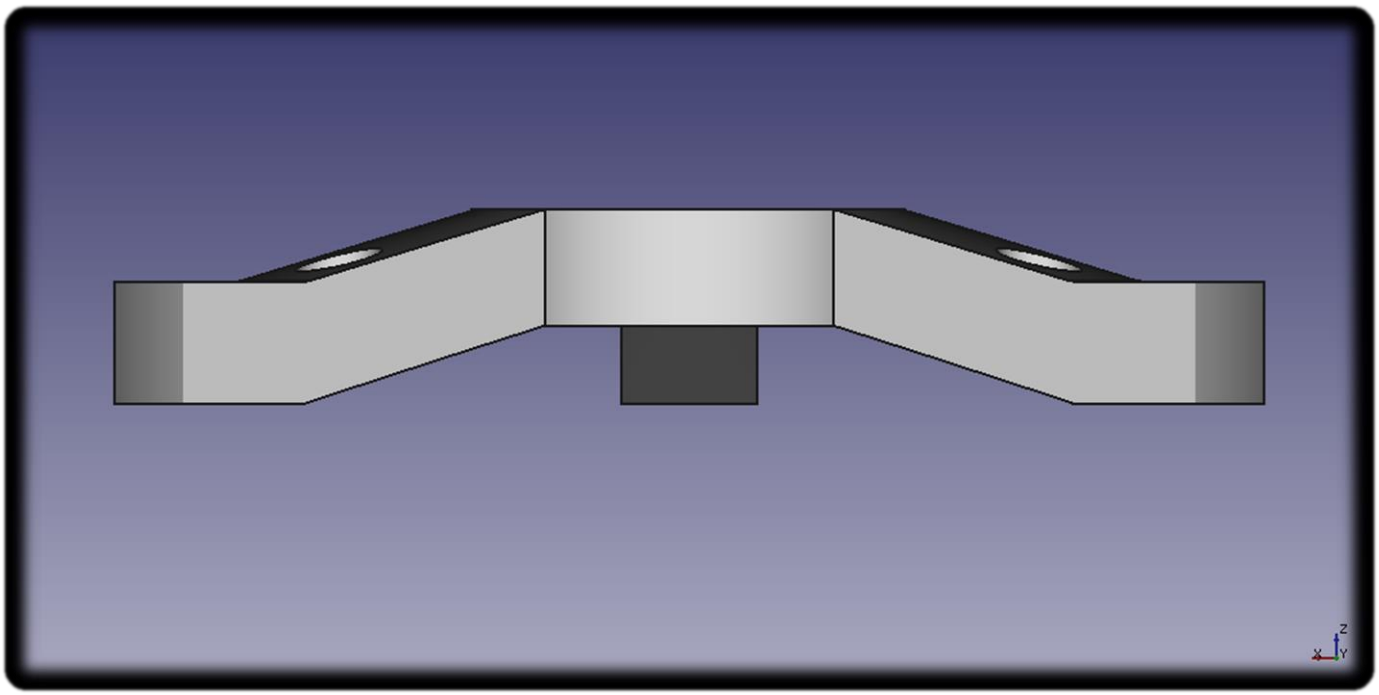


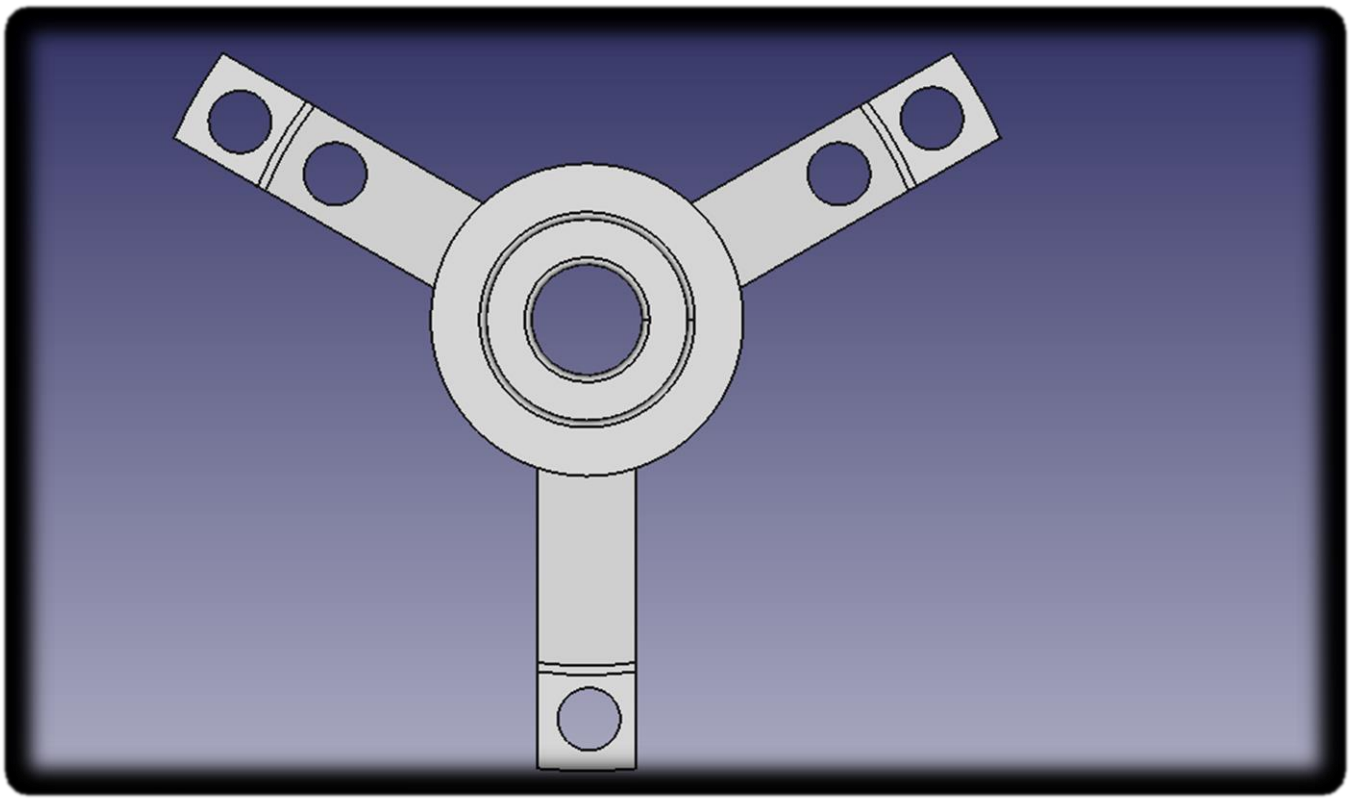
The Chromatography column contains eight main parts: top, handle, cylinder, piston, disc, bottom, base, and column.

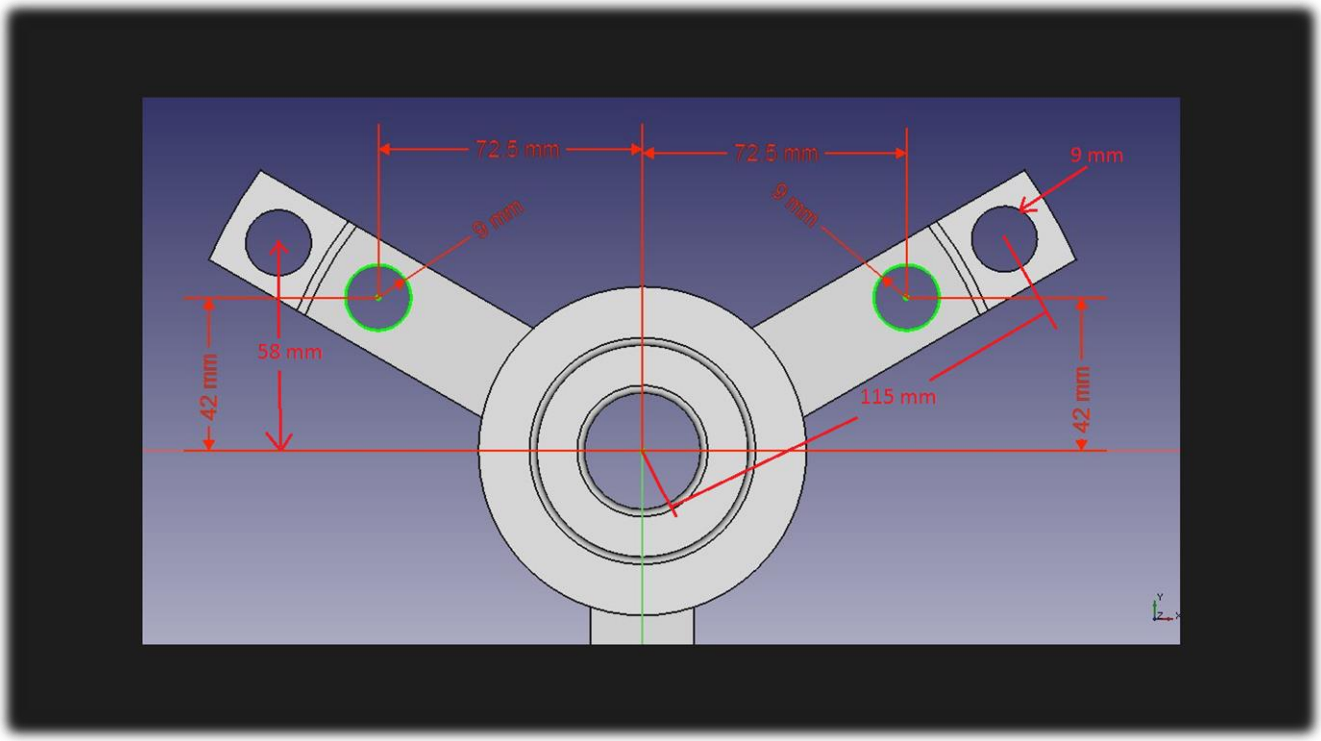
15.4.2.1 Top

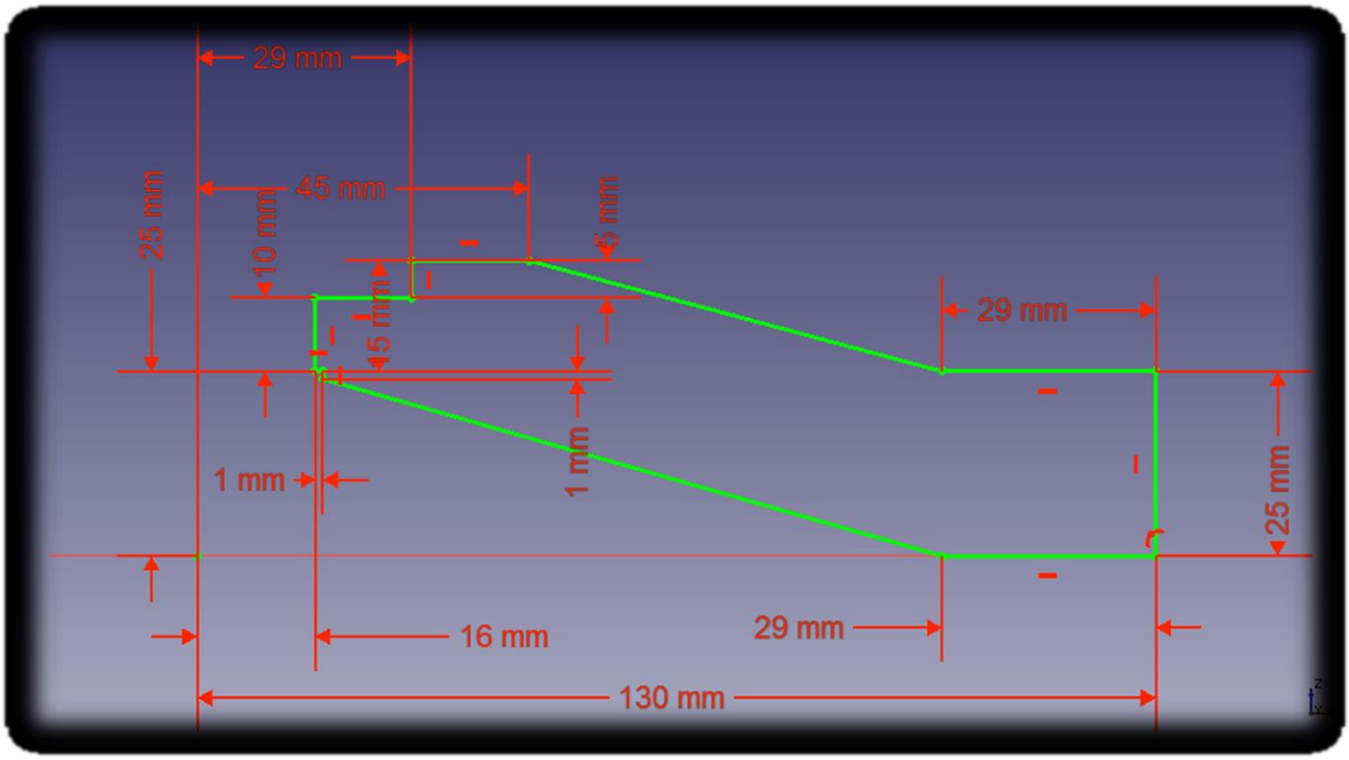
The top is fixed by nuts with three columns that pass through it, and it contains two other holes to let the tube of the piston pass through it







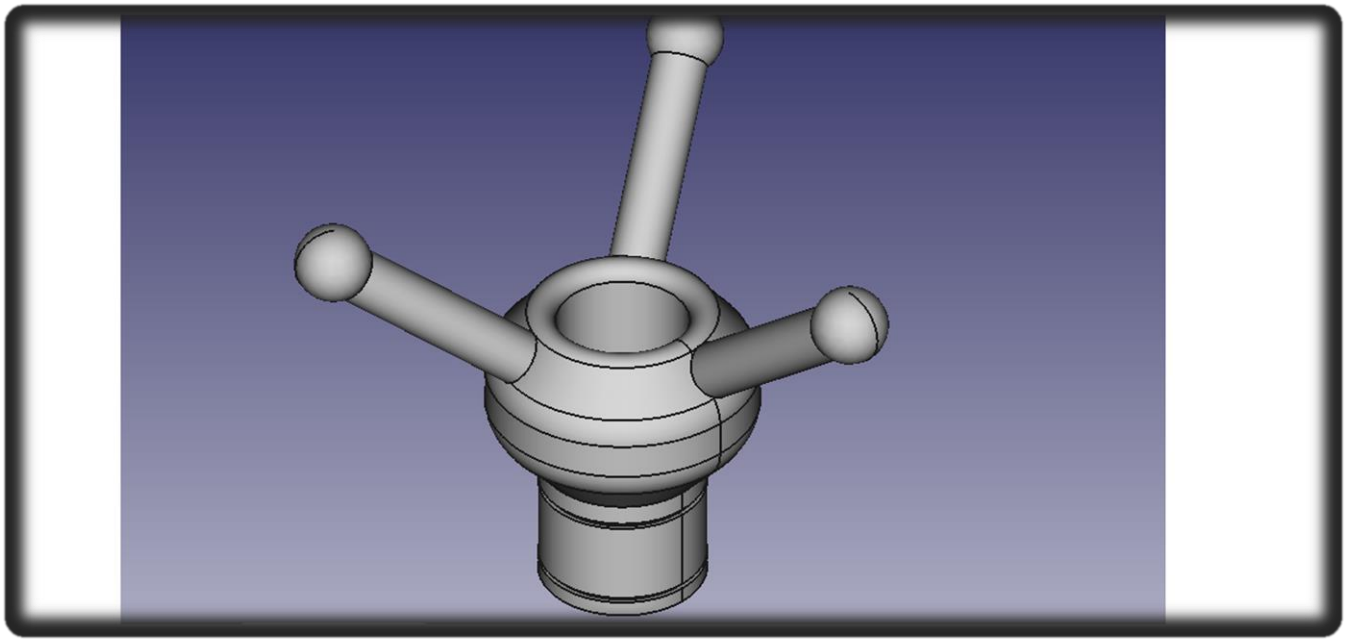


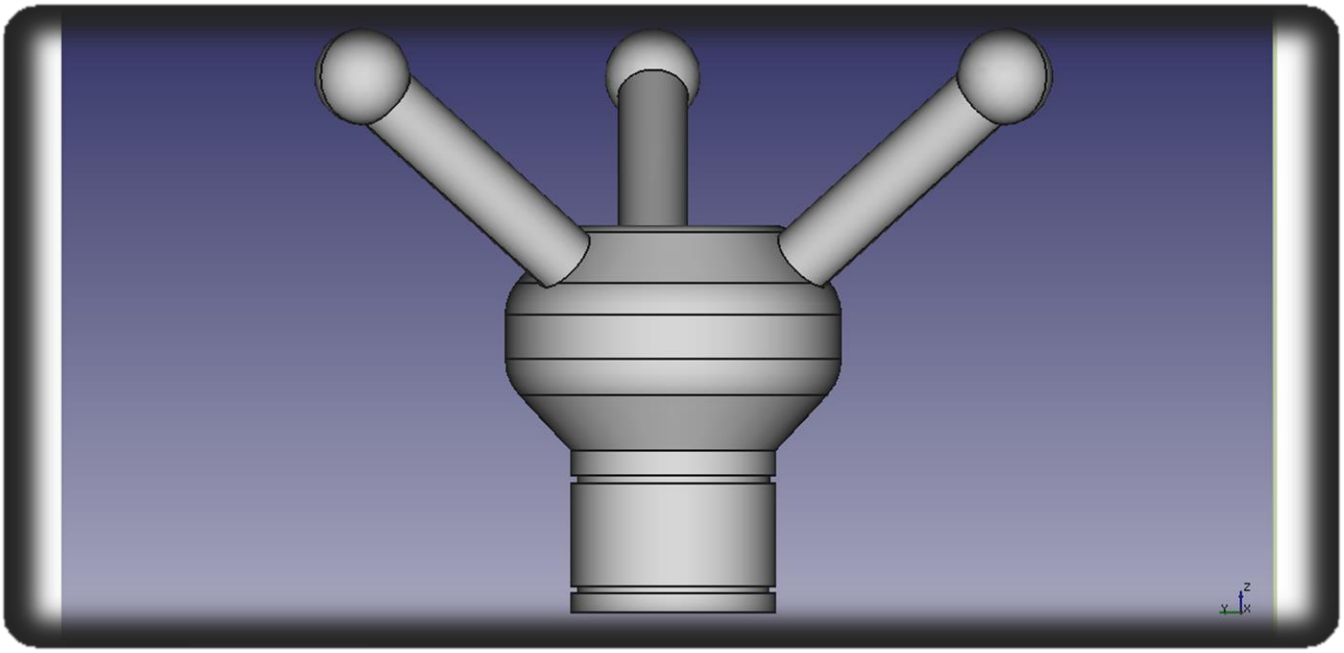


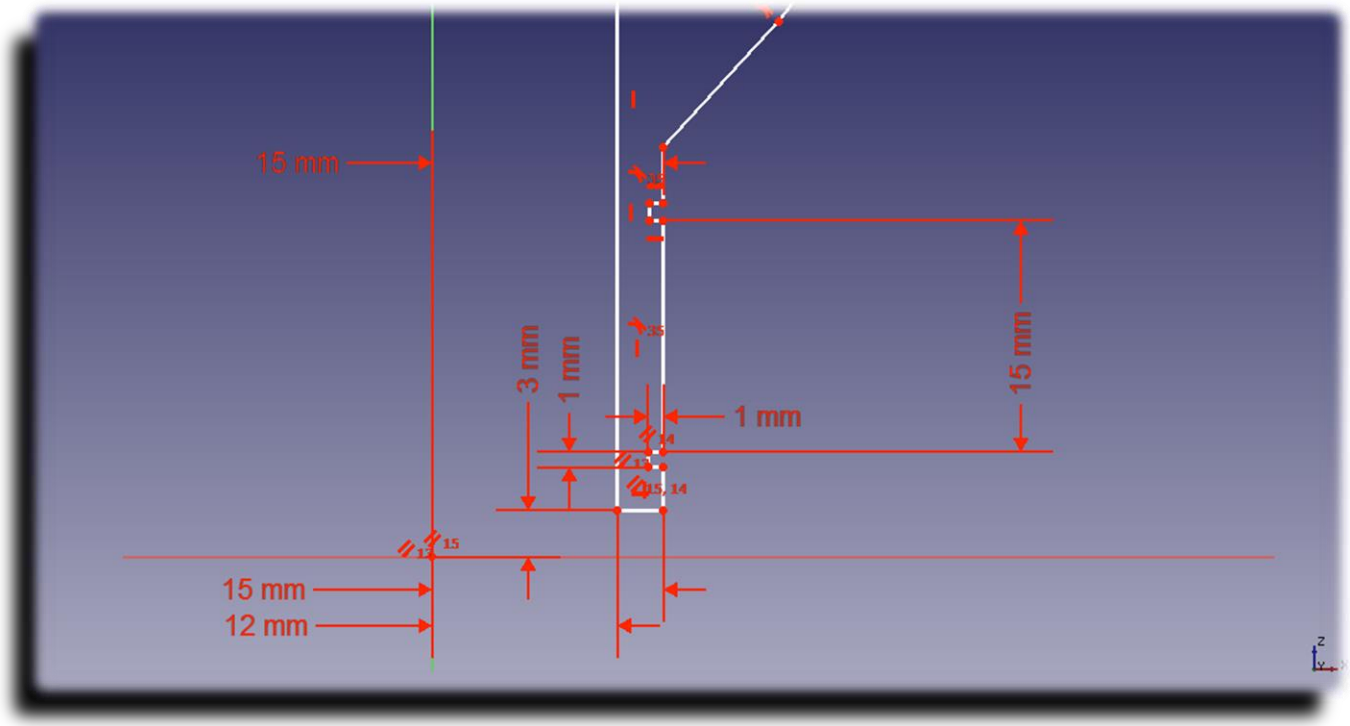
15.4.2.2 Handle

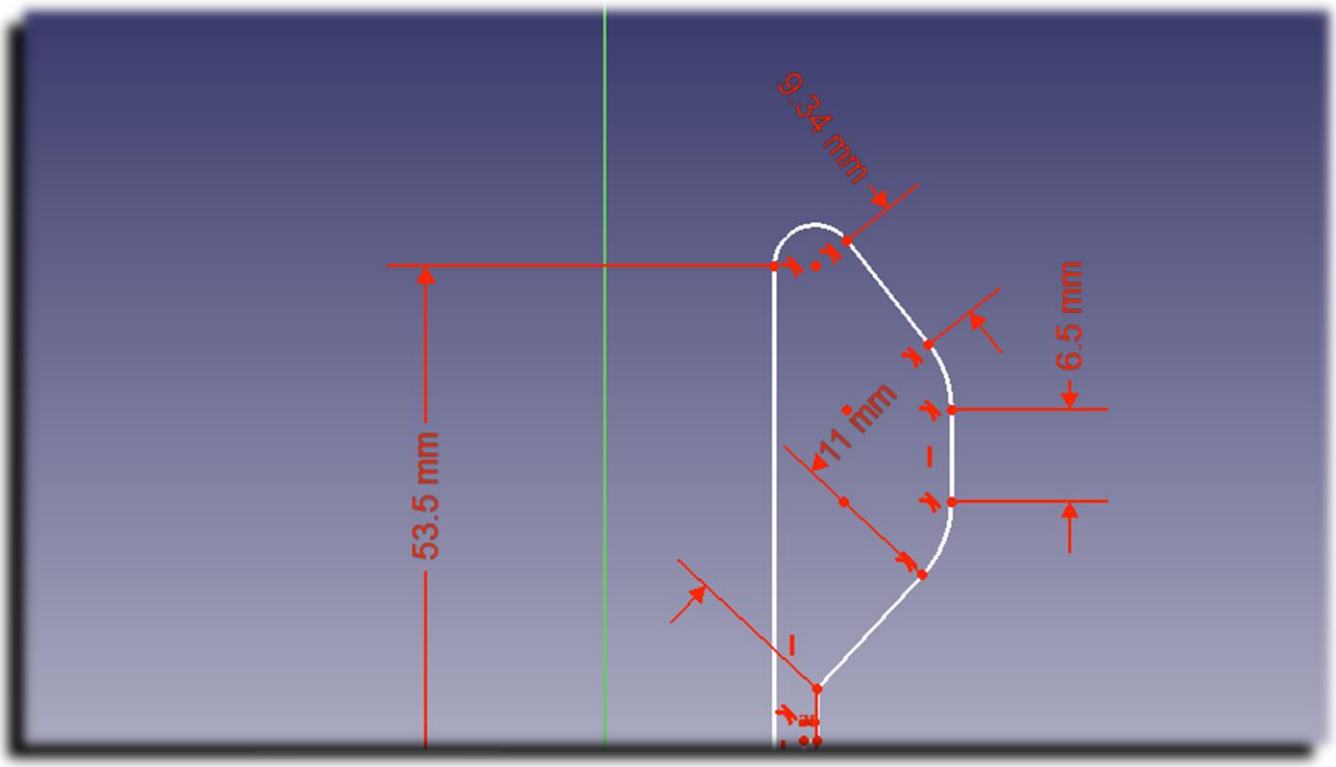
I used a handle instead of a motor, because it doesn't need a high power to rotate it and it is cheaper

Bearing is placed on the bottom between two rings



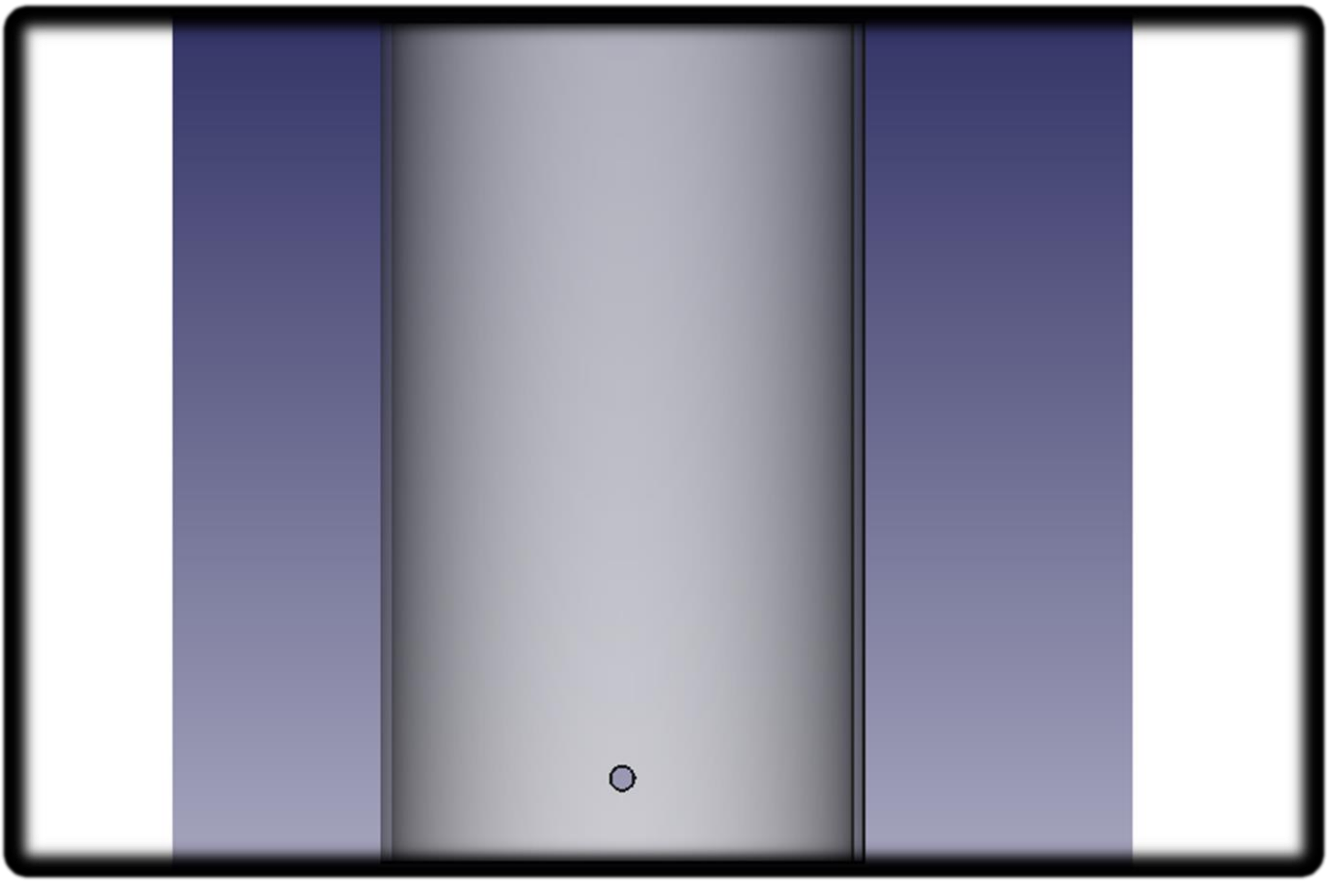


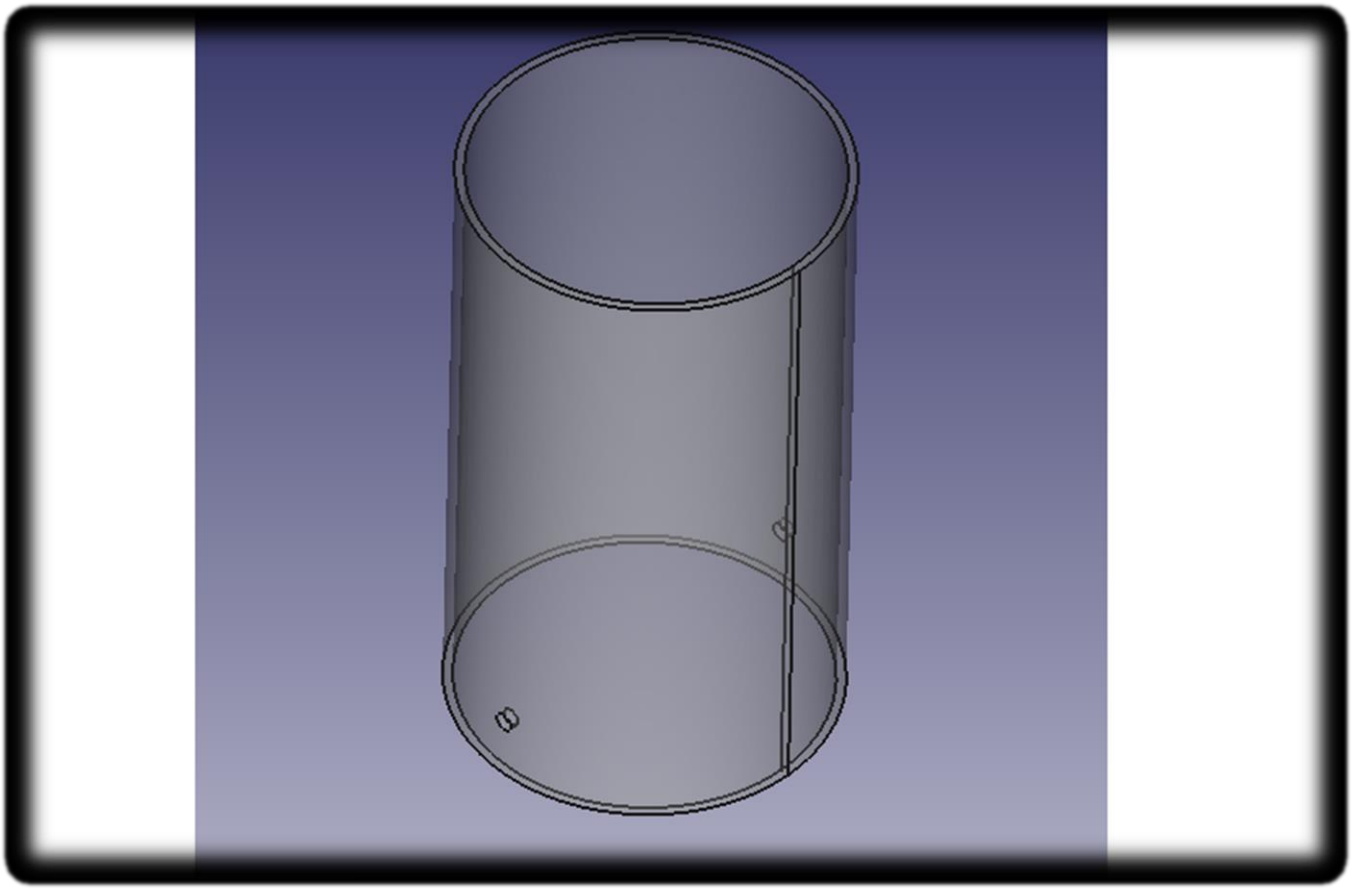


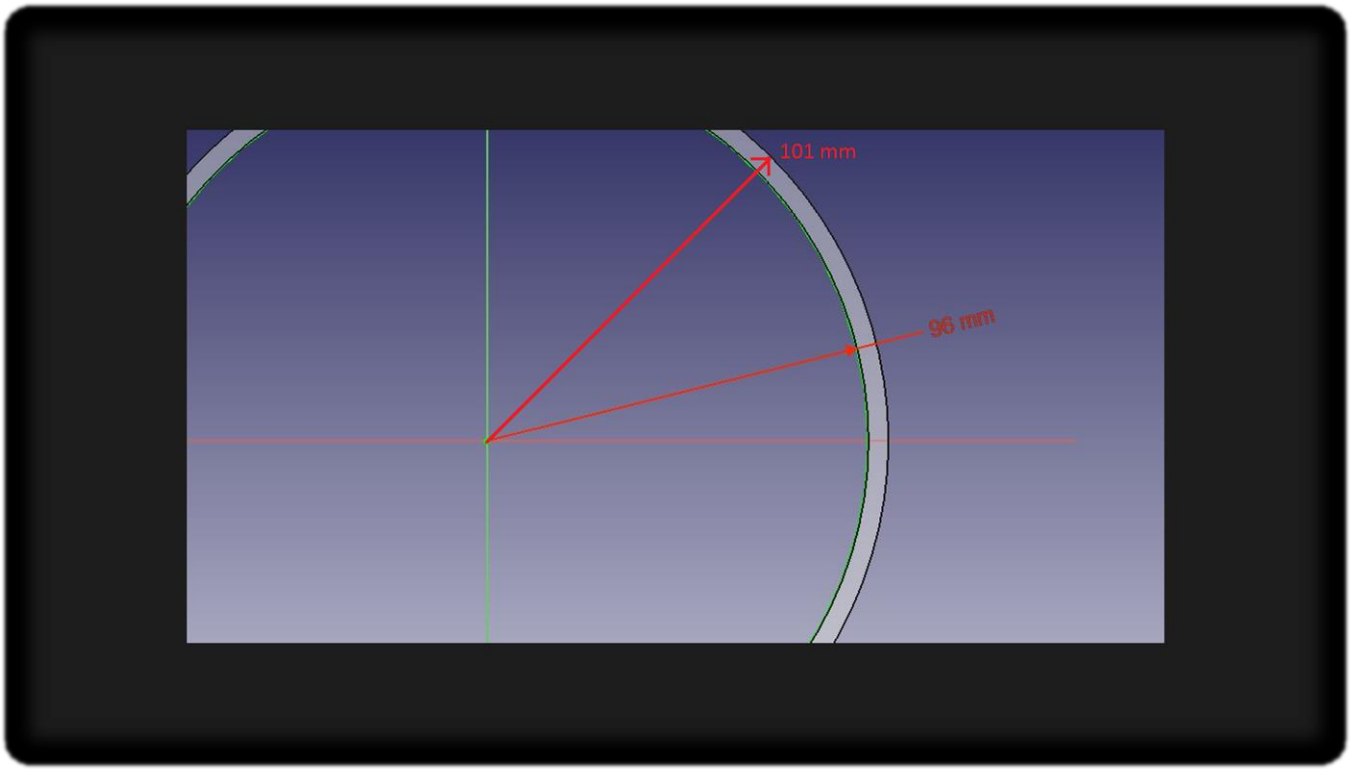


15.4.2.3 Cylinder

Is made of Plexiglas, it's where we put the solid particles

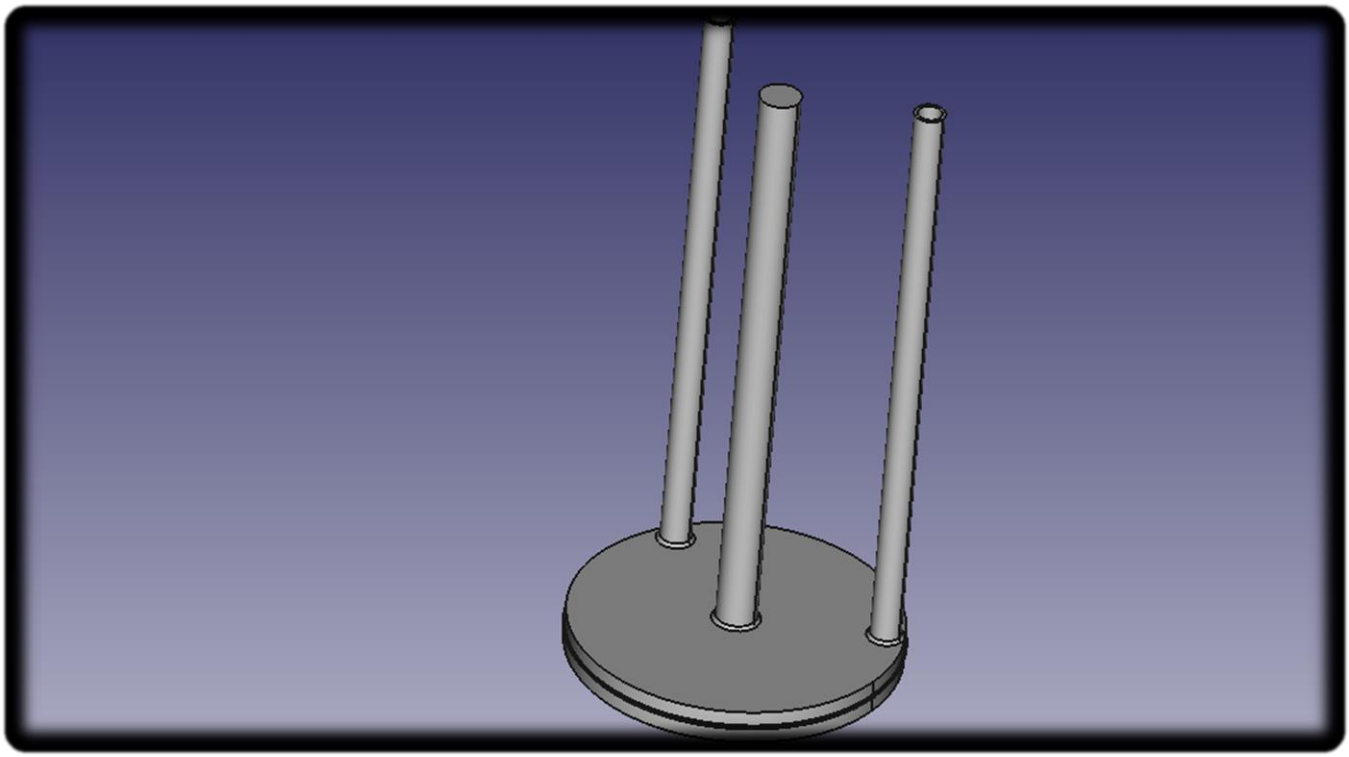


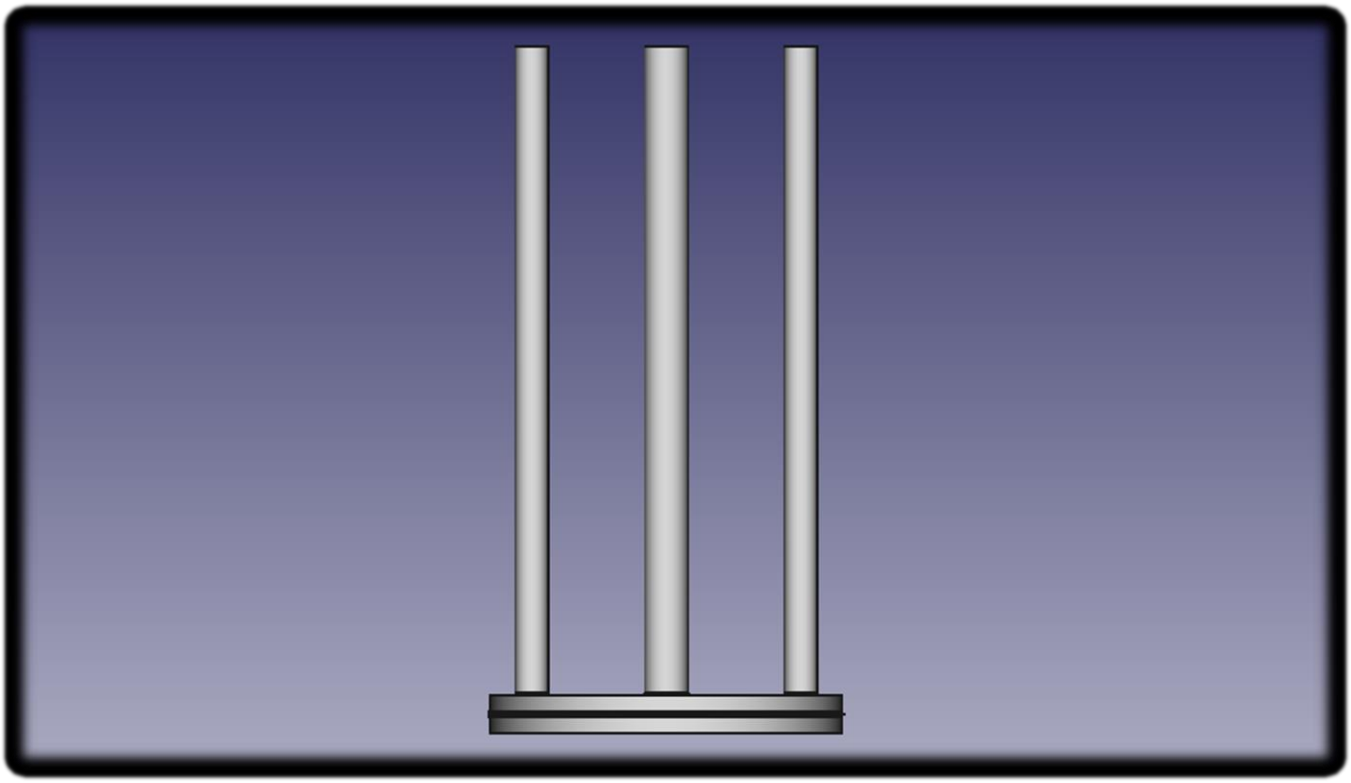


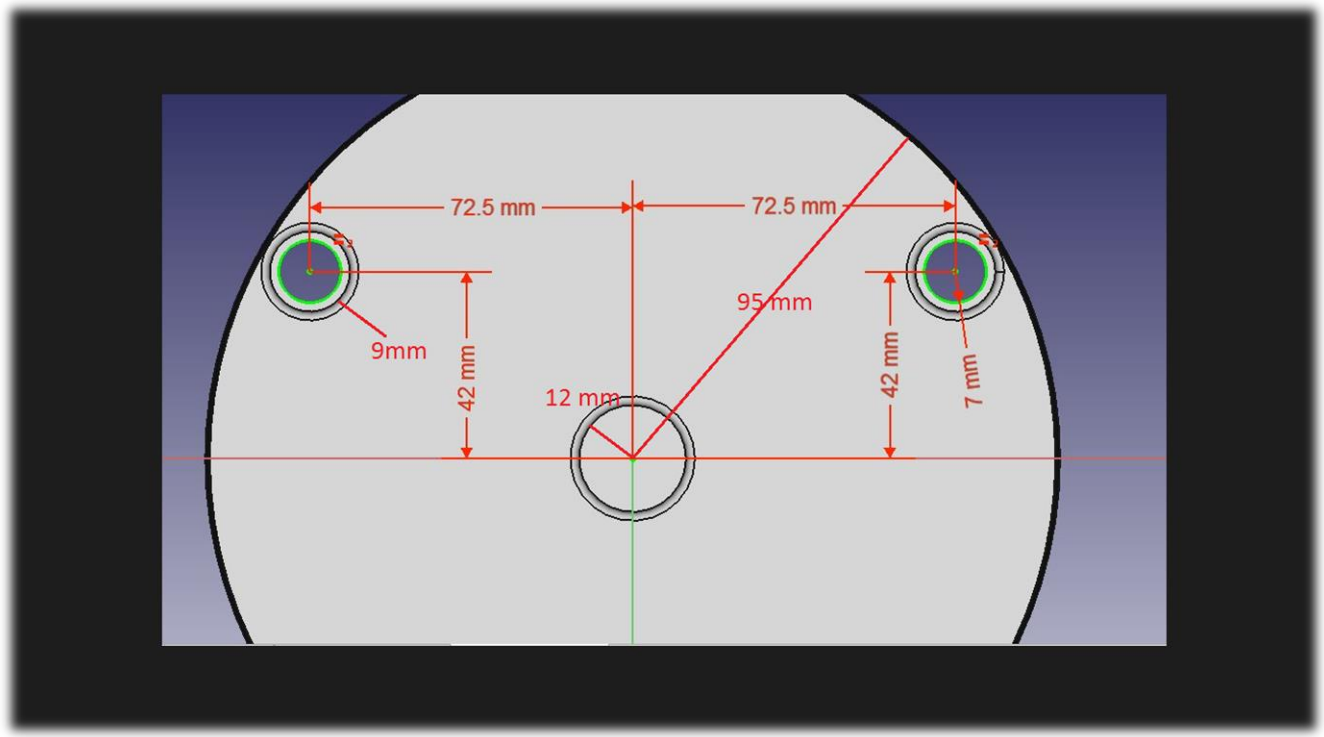


15.4.2.4 Piston

Contains a ring to avoid fluid leaks and to let the piston move easily and two tubes where fluid passes through into the piston

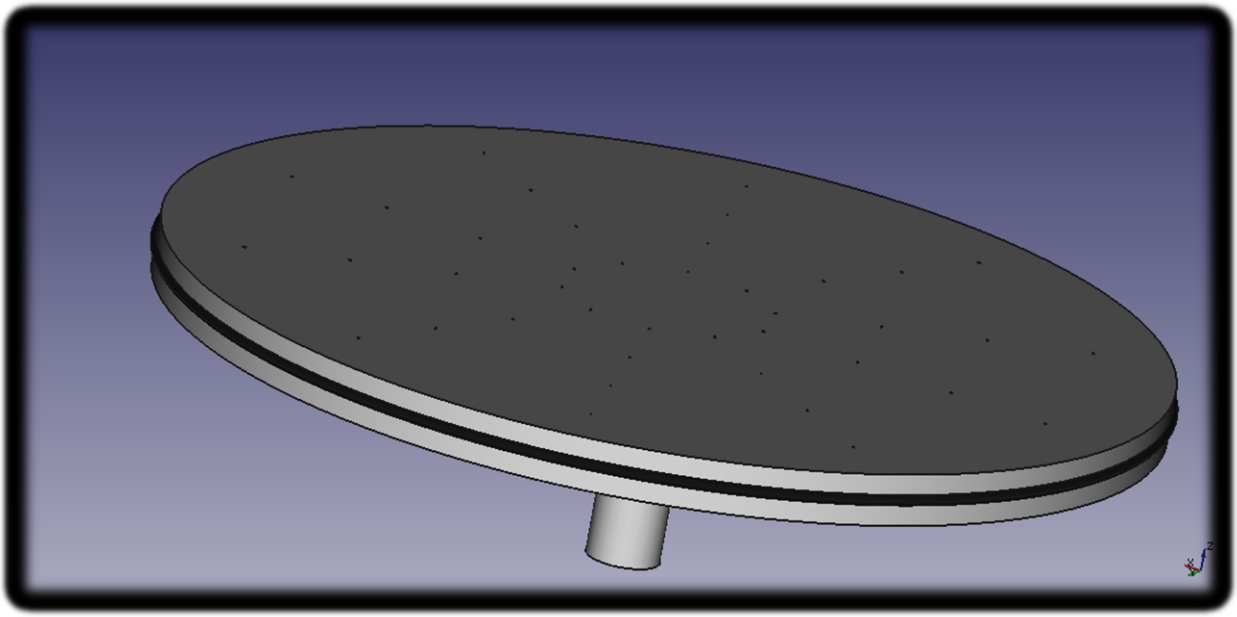


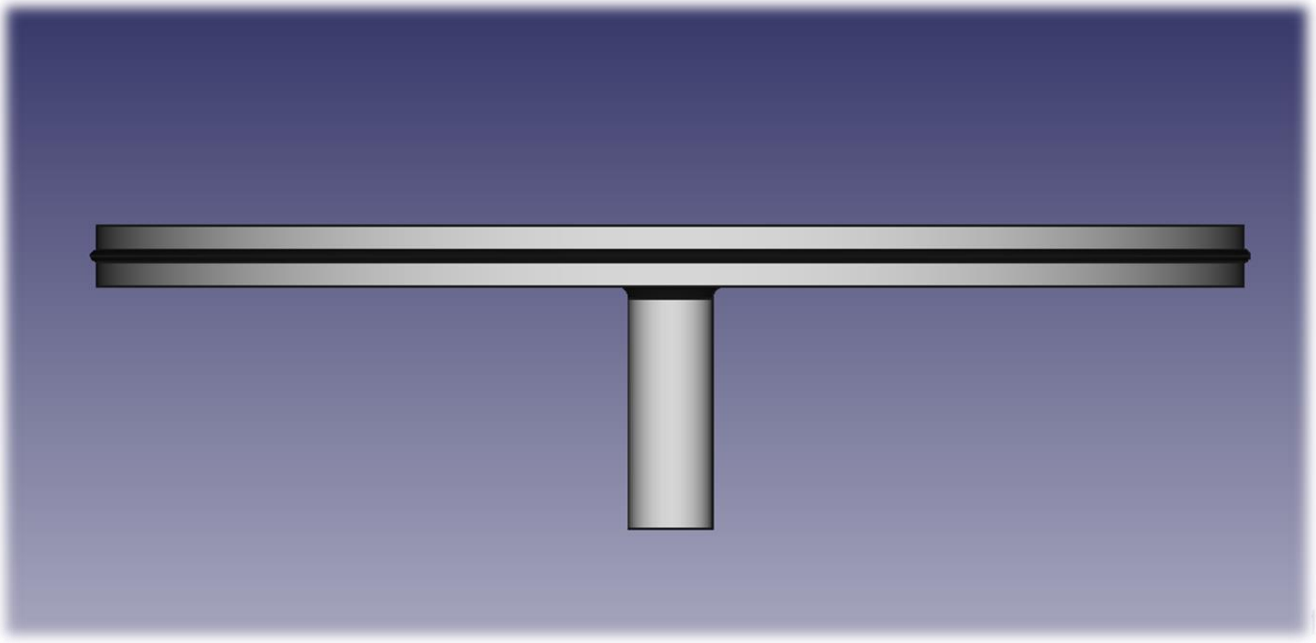


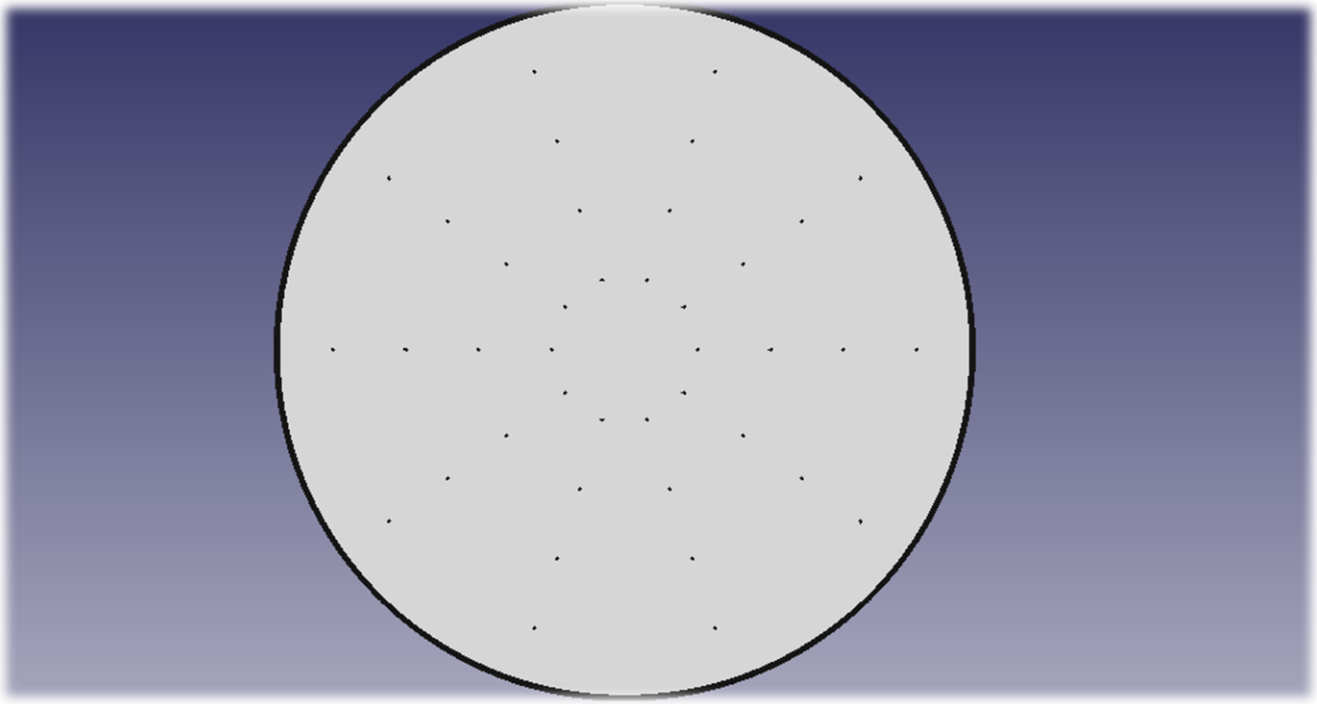

72.5 mm
72.5 mm
95 mm
9mm
42 mm
12 mm
42 mm
7 mm

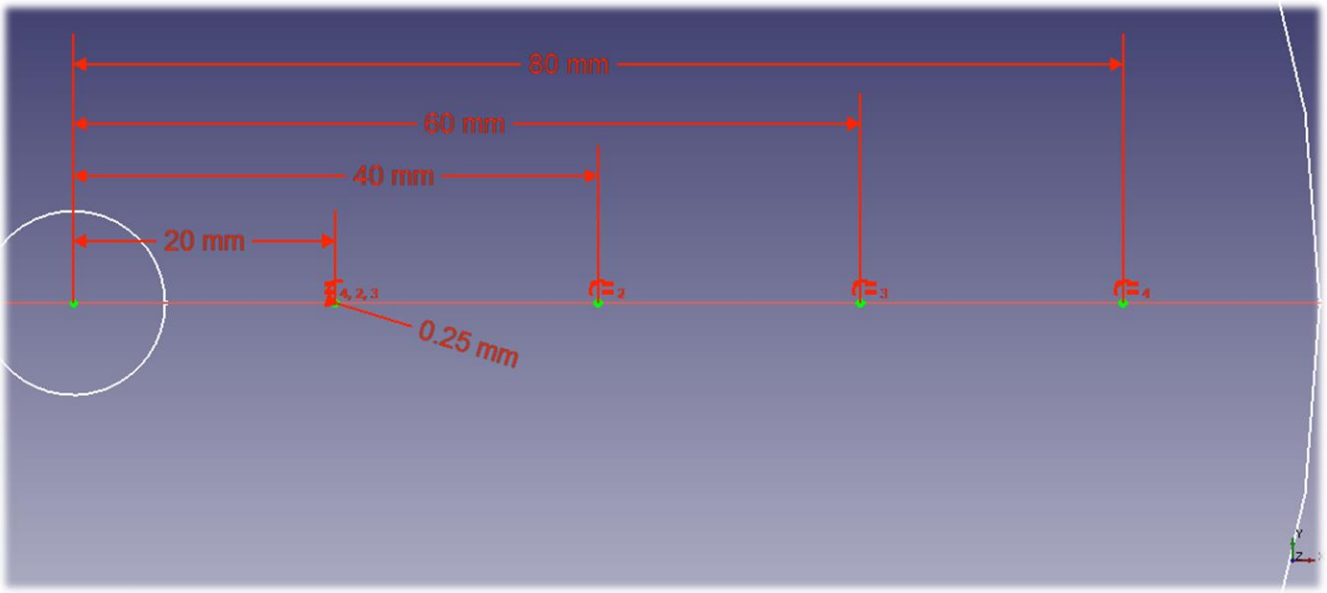
15.4.2.5 Disc

Is placed on the bottom of the cylinder. It contains holes of diameter 0.25 mm to separate big molecules from small ones and it contains a ring to avoid leakage



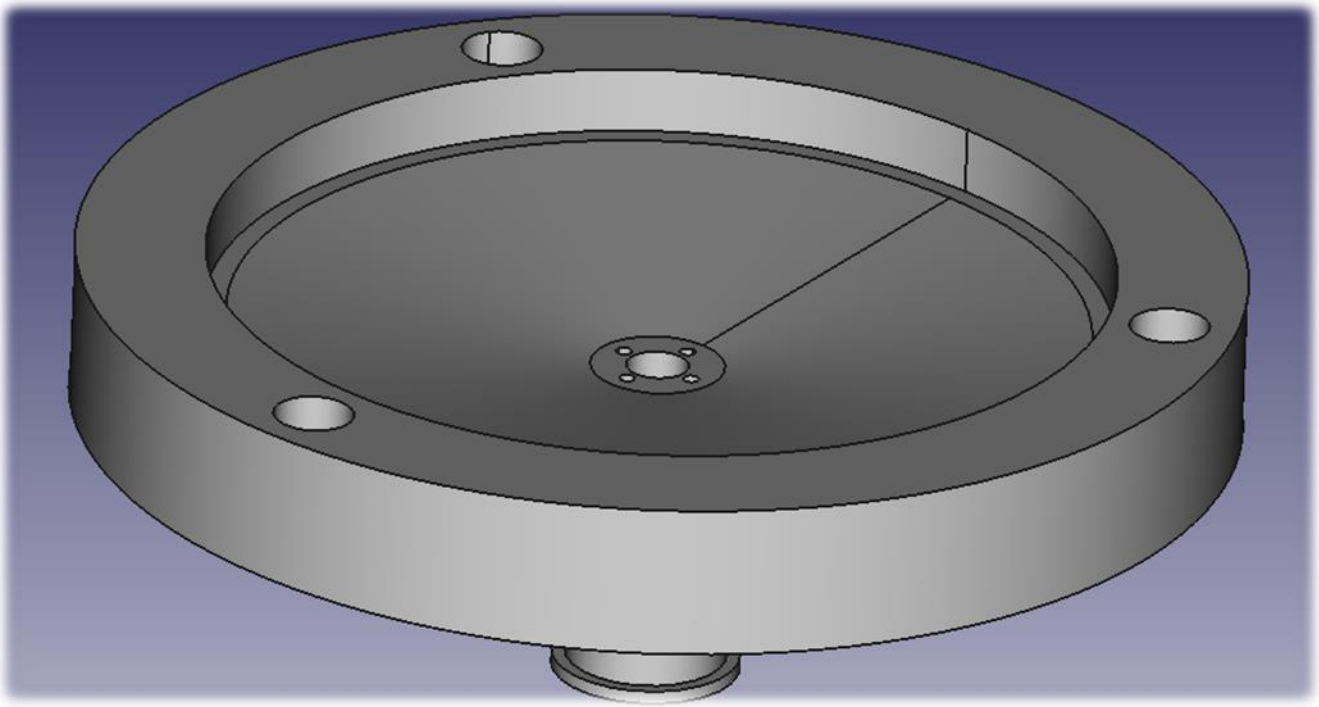


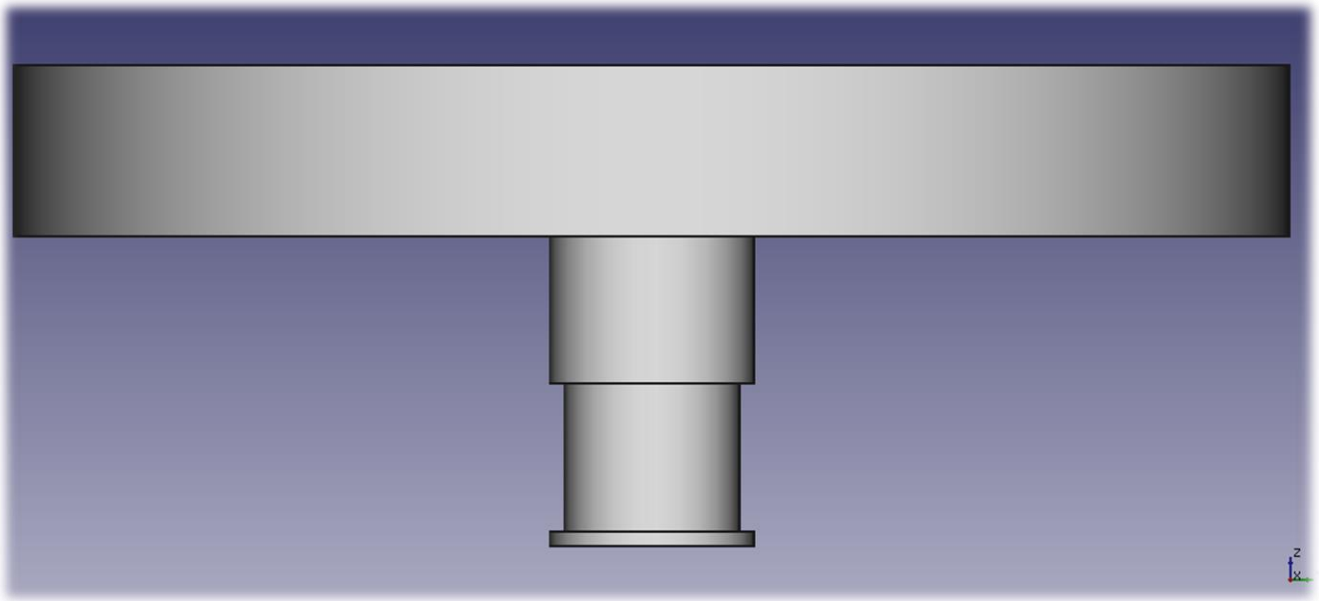


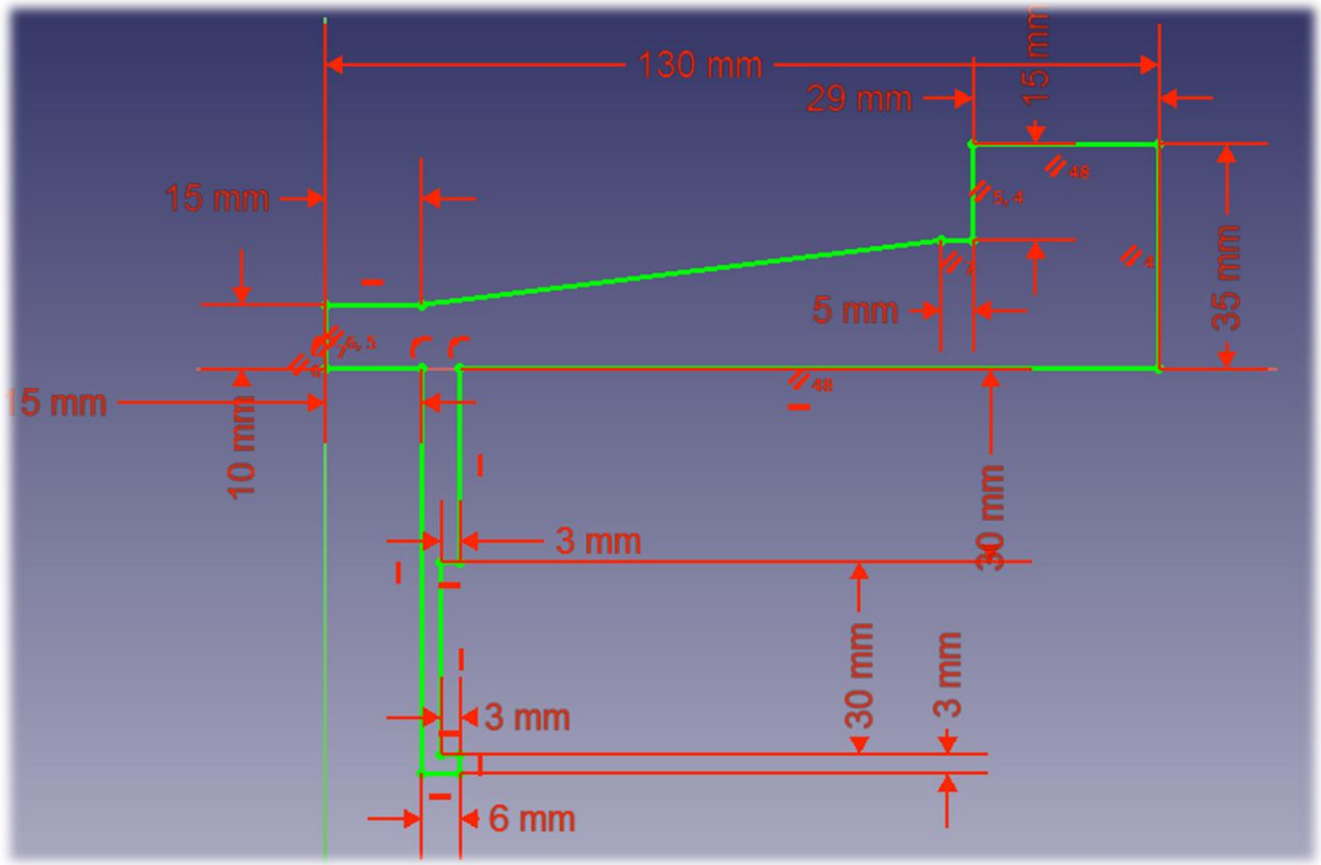

80 mm
60 mm
40 mm
20 mm
0.25 mm

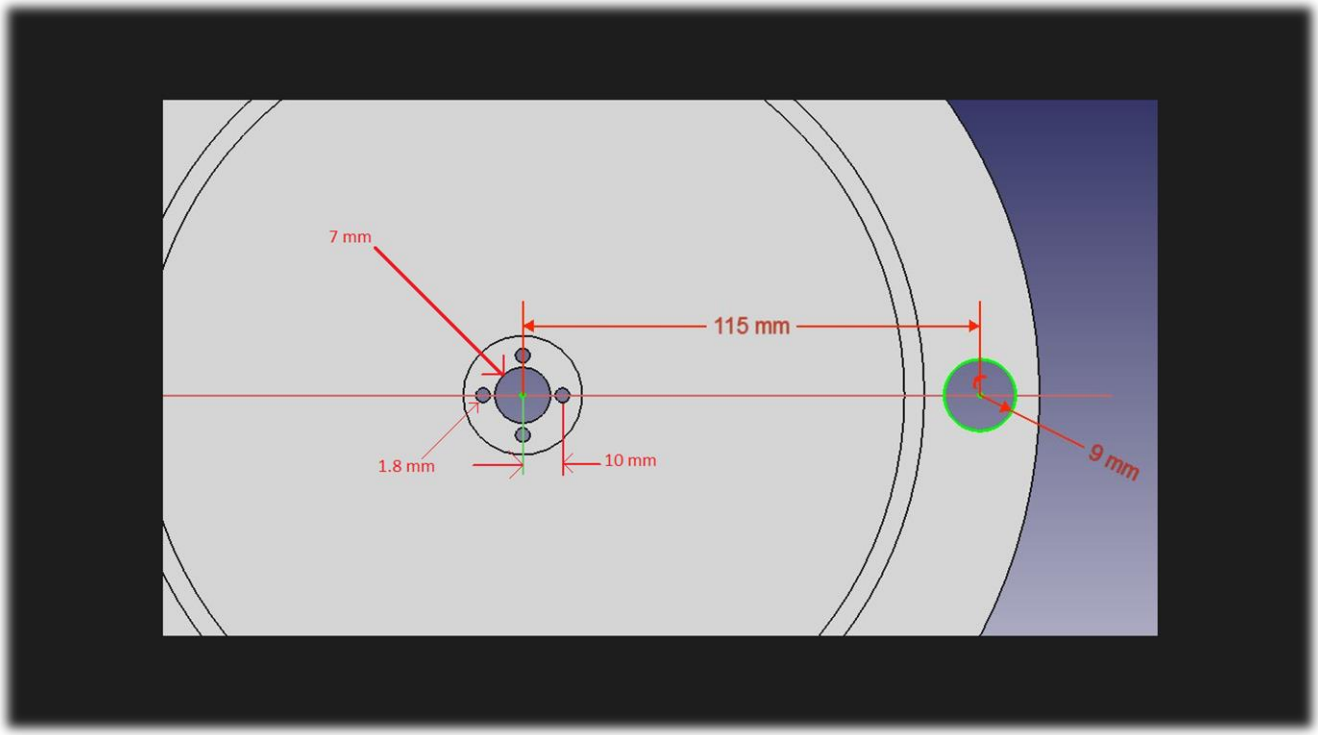
15.4.2.6 Bottom

Contains three external holes fixed to the three columns by bolts.it has a conical shape to let the fluid reach to the center



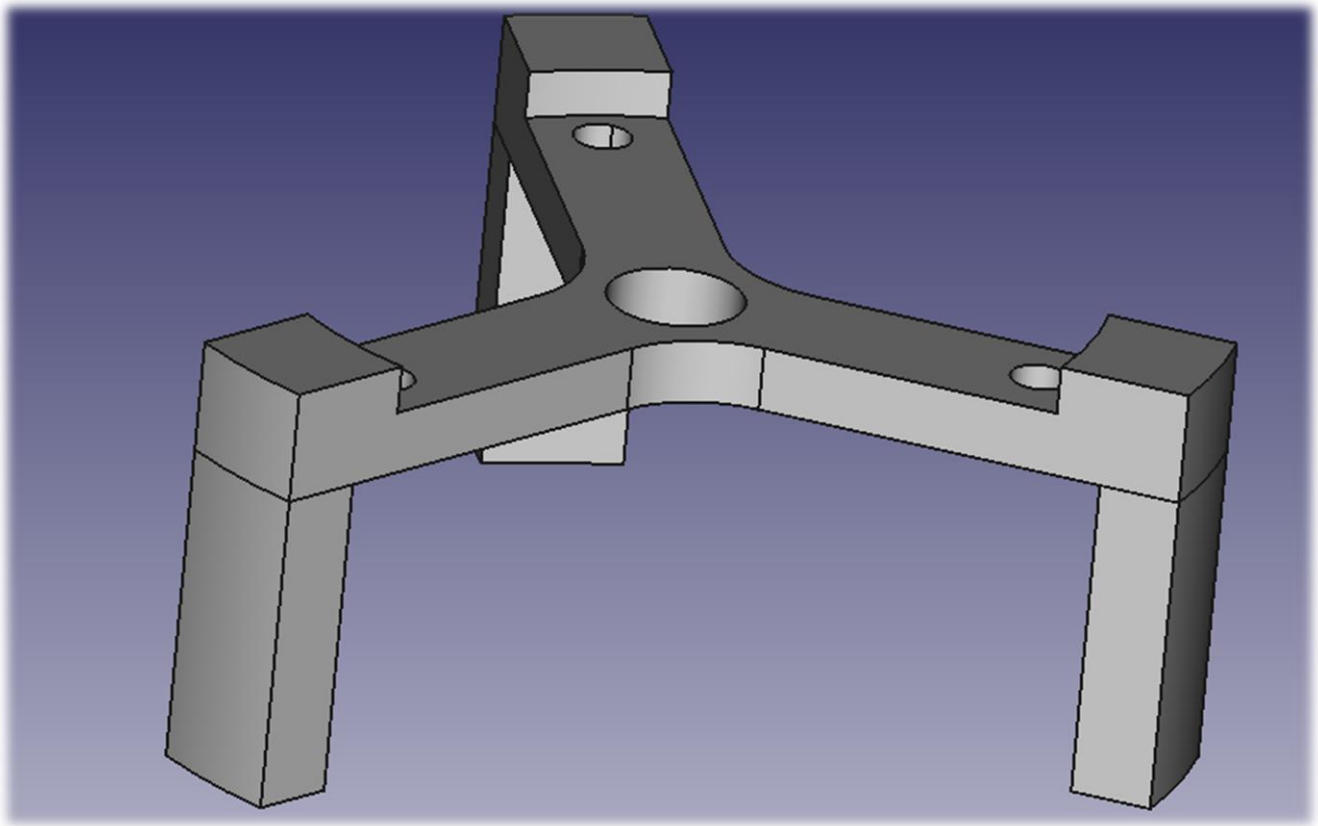


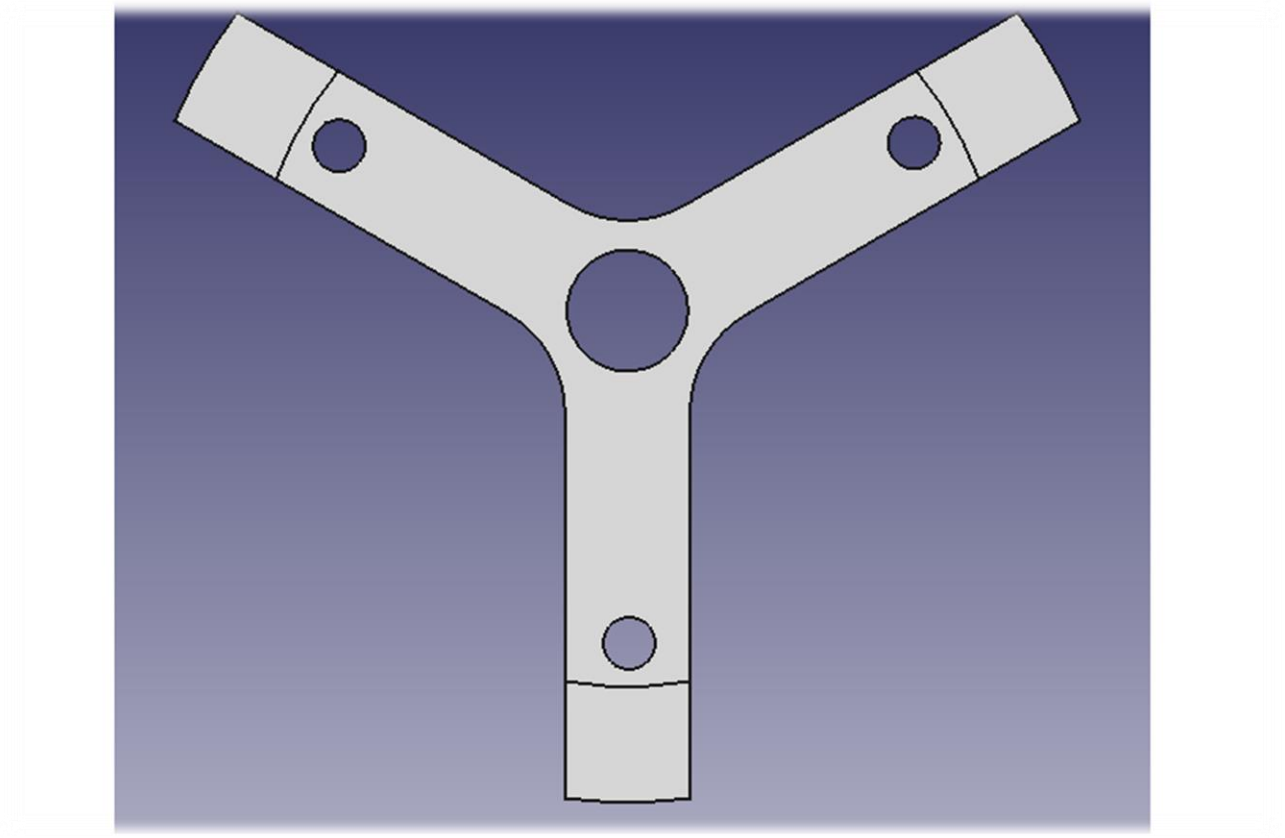

130 mm
29 mm
15 mm
15 mm
35 mm
5 mm
15 mm
10 mm
3 mm
30 mm
30 mm
3 mm
3 mm
6 mm

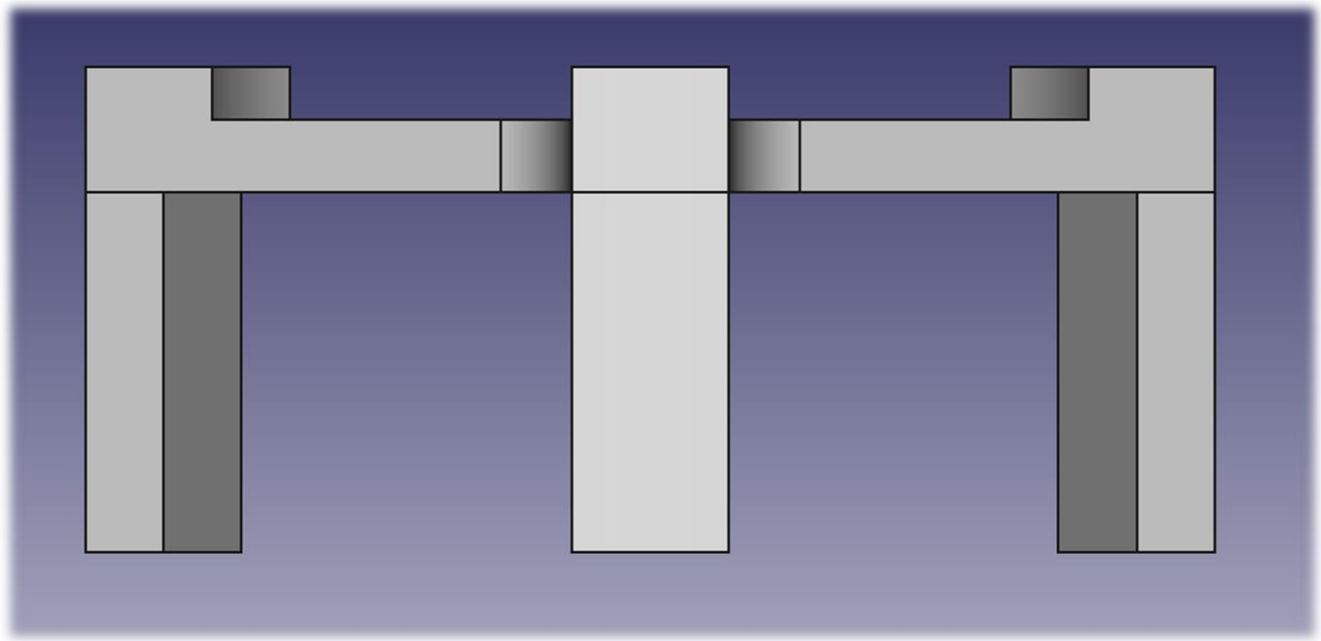


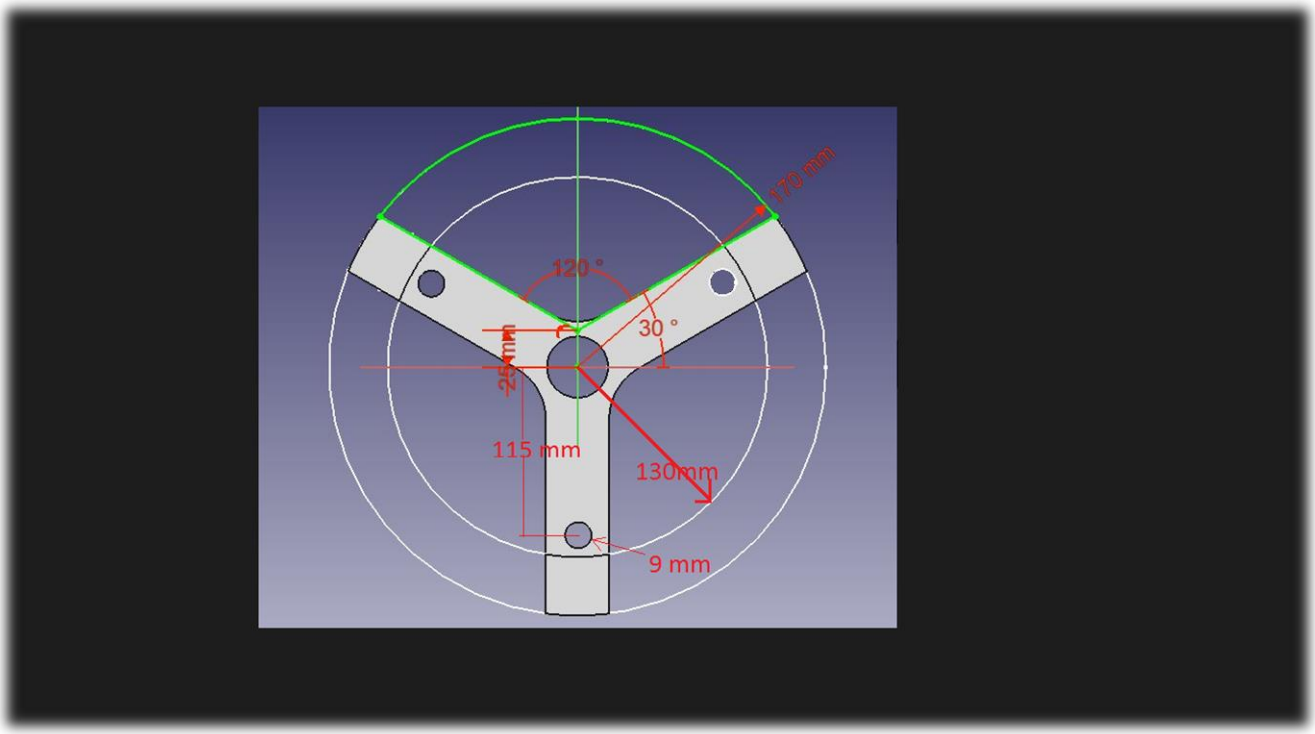
15.4.2.7 Base

Contains three legs and three external holes to fix the columns by bolts



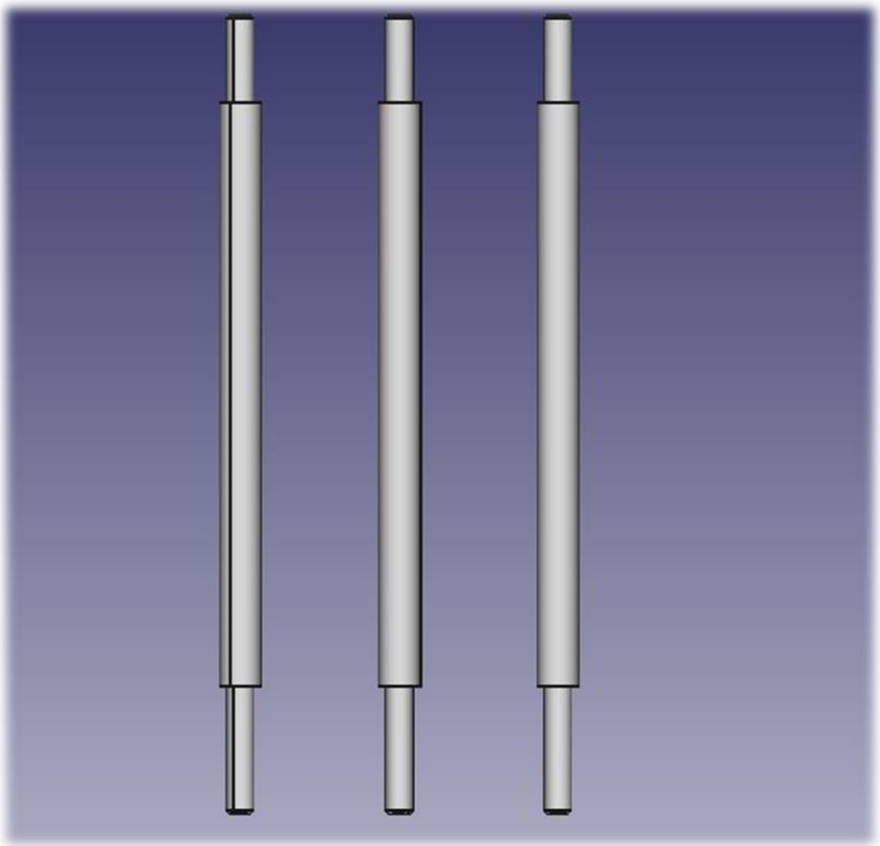


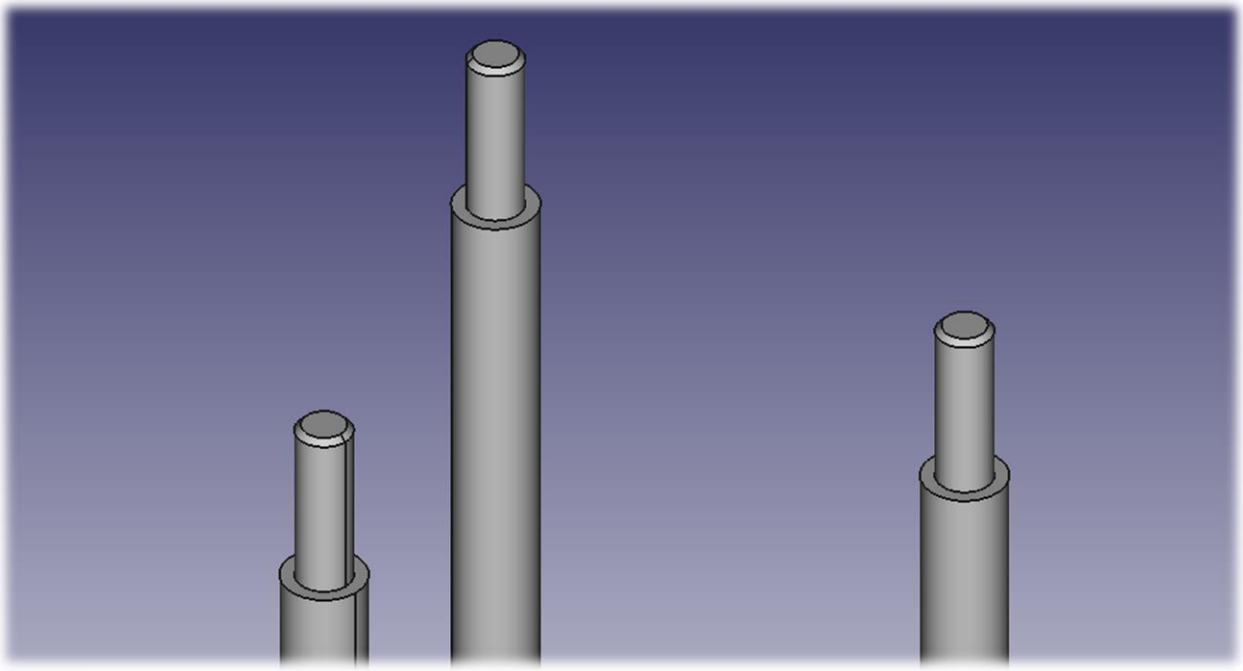


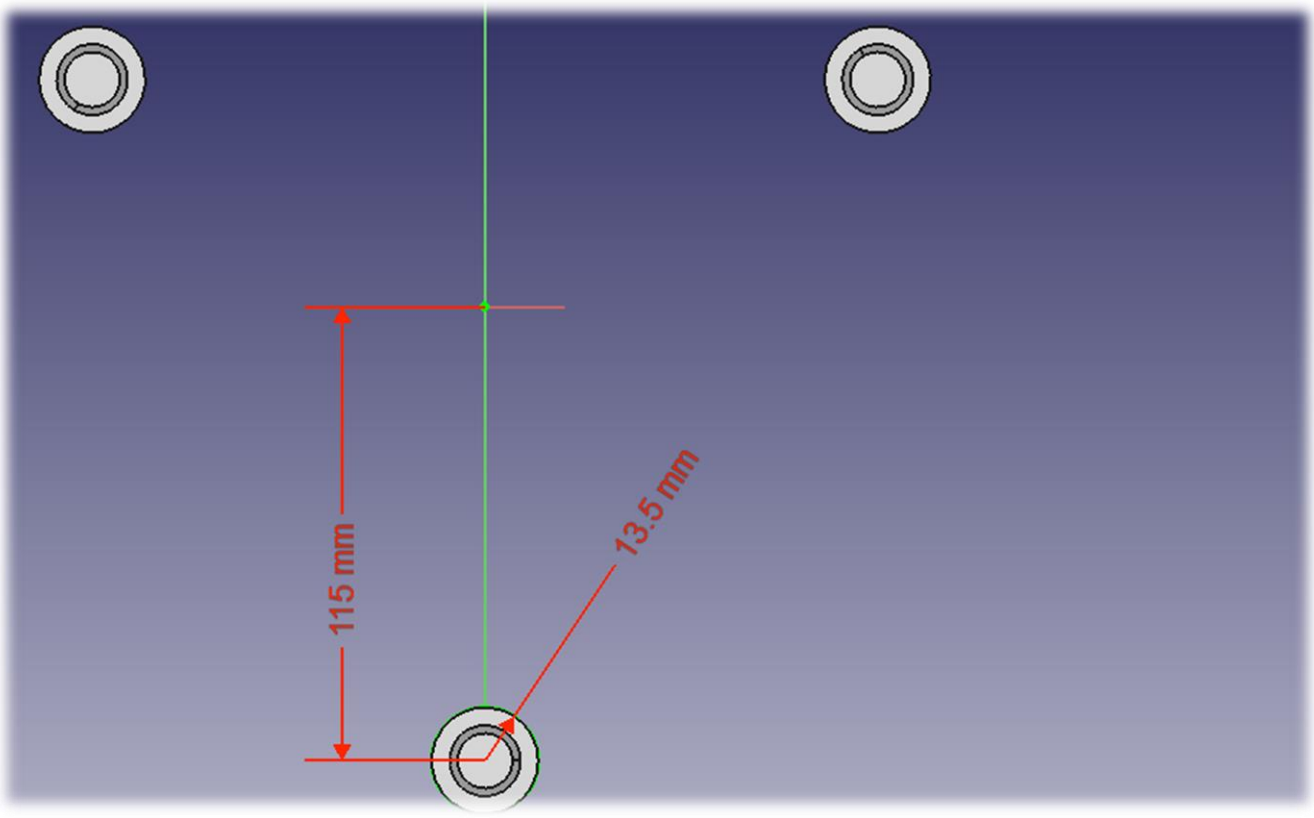


15.4.2.8 Column

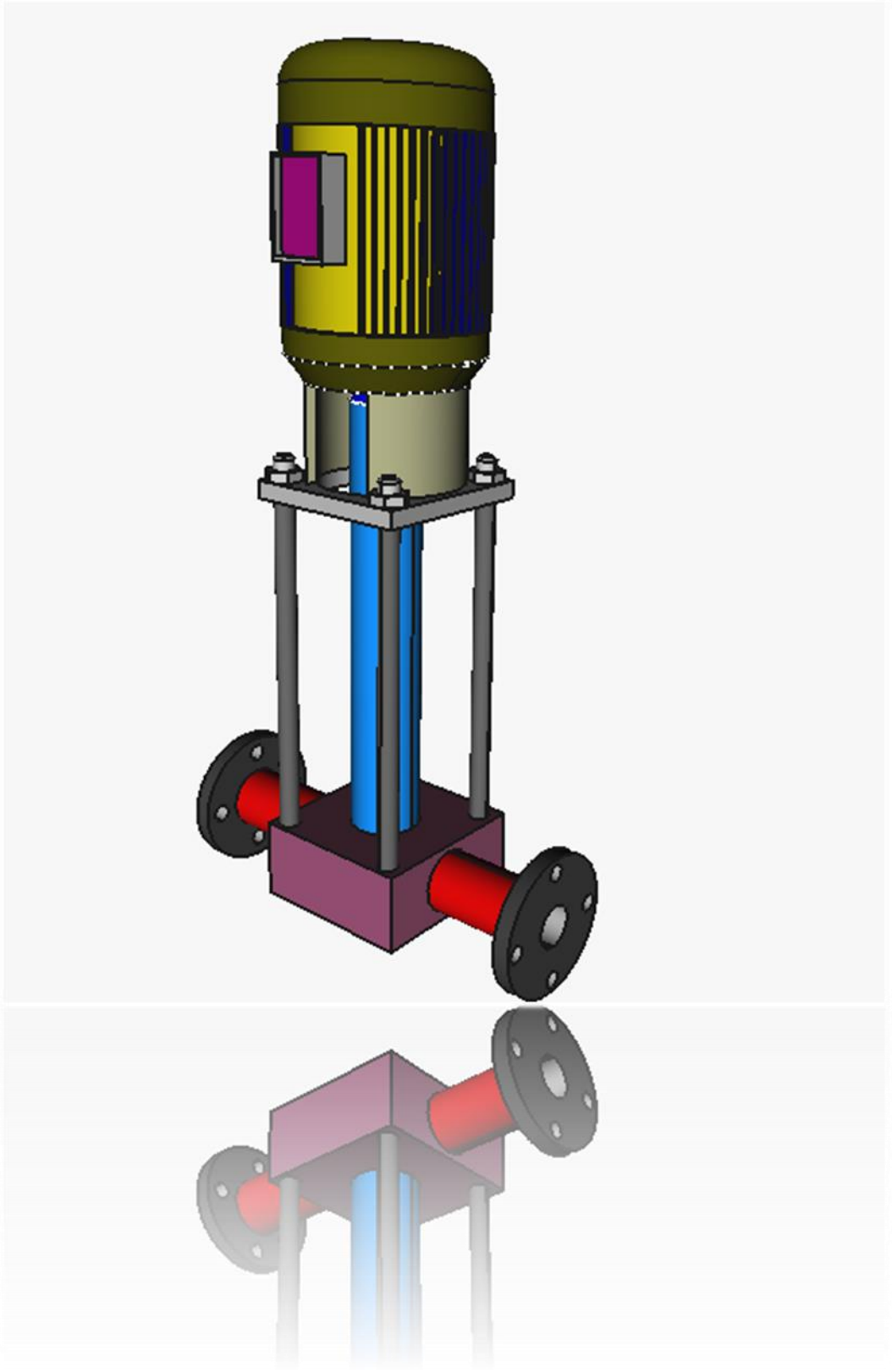
It is the part that connects the top, bottom and base parts together.

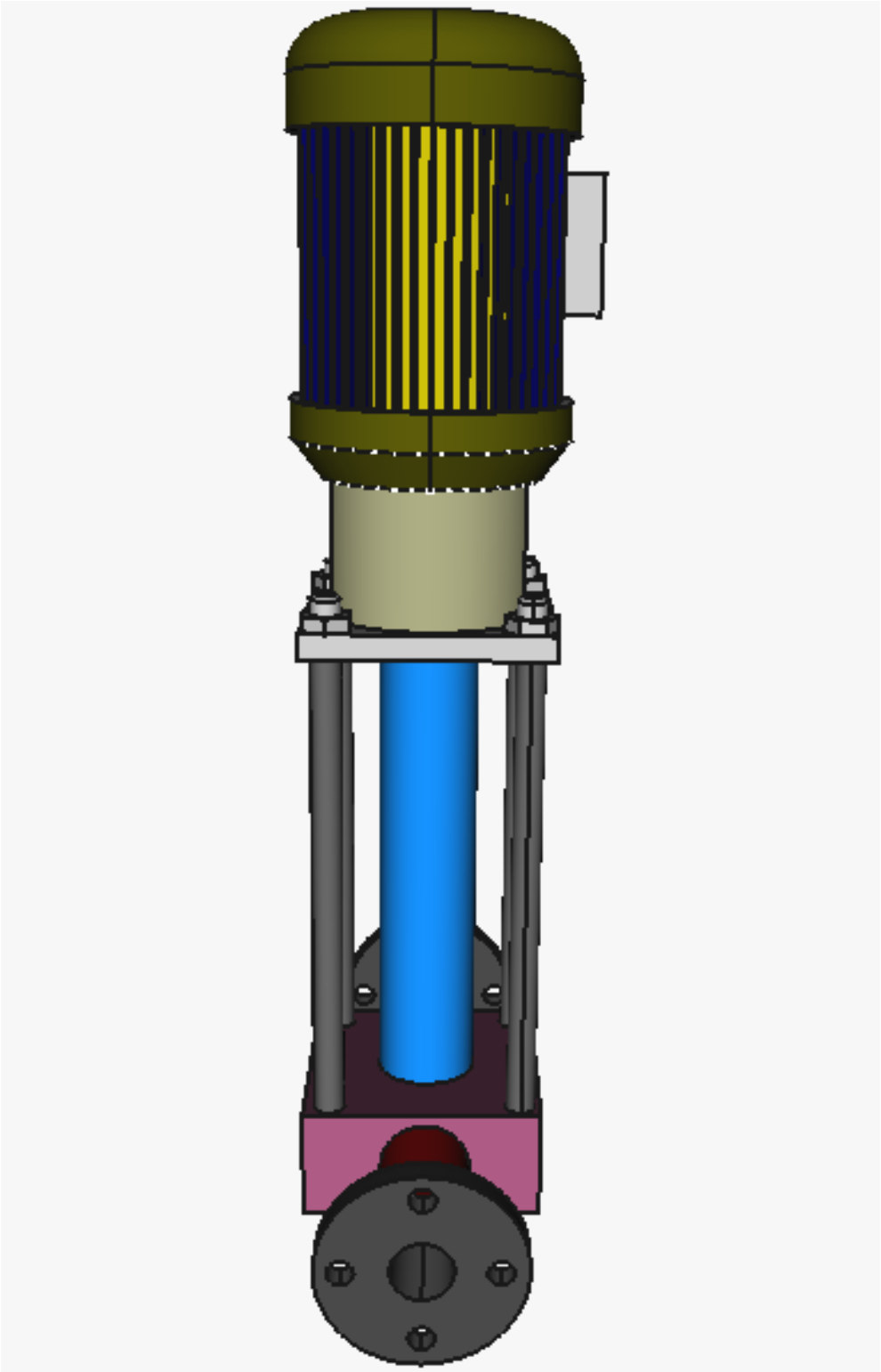


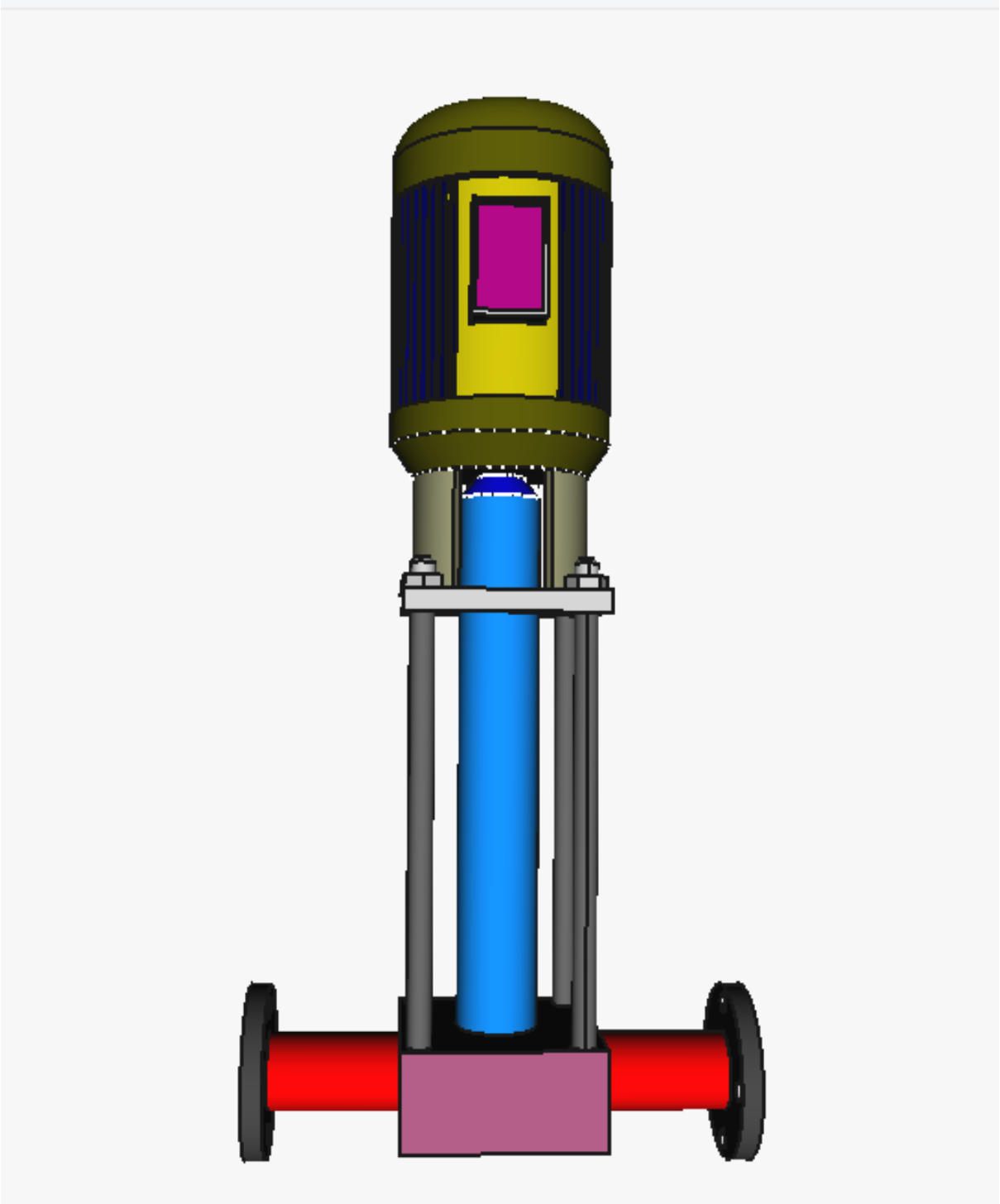



115 mm
13.5 mm

15.5 Demonstration and Modelling of the Pump







15.6 Demonstration and Modelling of the Homogenizer

-Ibrahim Zaaroura-

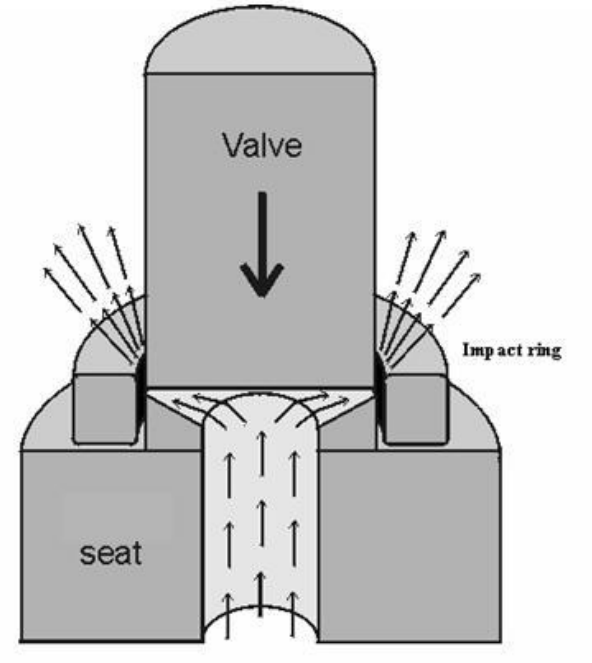
15.6.1 DEVICE DETAILS & SPECIFICATIONS

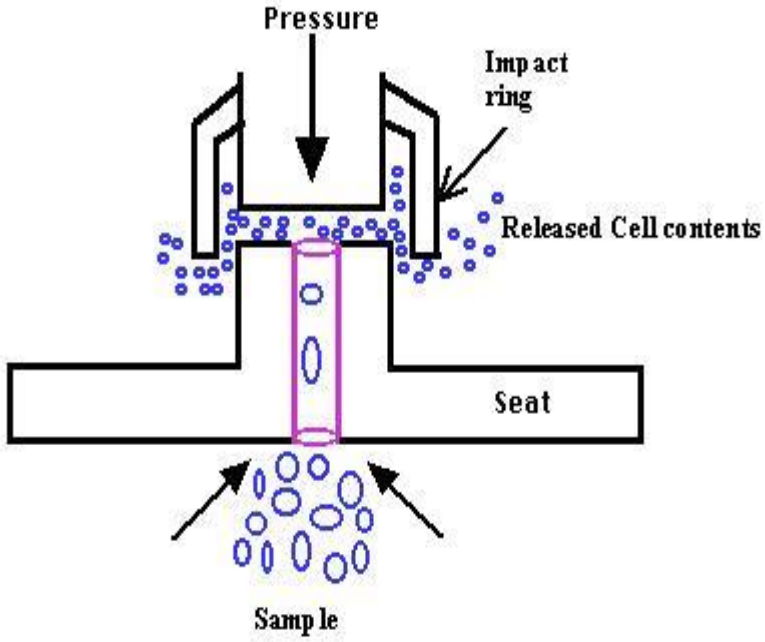
A homogenizer is a piece of laboratory or industrial equipment used for the homogenization of various types of material, such as tissue, plant, food, soil, and many others. Many different models have been developed using various physical technologies for disruption.

Homogenization is a very common sample preparation step prior to the analysis of nucleic acids, proteins, cells, metabolism, pathogens, and many other targets.

15.6.1.1 Working principle

High Pressure Homogenization is a process of increasing the consistency of a product by means of dispersions. The product is displaced under the generation of high pressure and is forced through homogenizing valve gap. Cavitation's turbulence and sheer force break the product into particles of size less than 1 microns.





Acting on high-pressure principle, the instrument is used for extruding cells, especially suitable for smashing thick-wall cells, germs and denser solution samples. Having no noise, less temperature rise and no 28 metal ion contamination, it has wide application of such research fields as protein study, nucleic acid extraction, cell disruption in genetic engineering labs of colleges, scientific research institutes and pharmaceutical factories.

15.6.1.2 Specifications

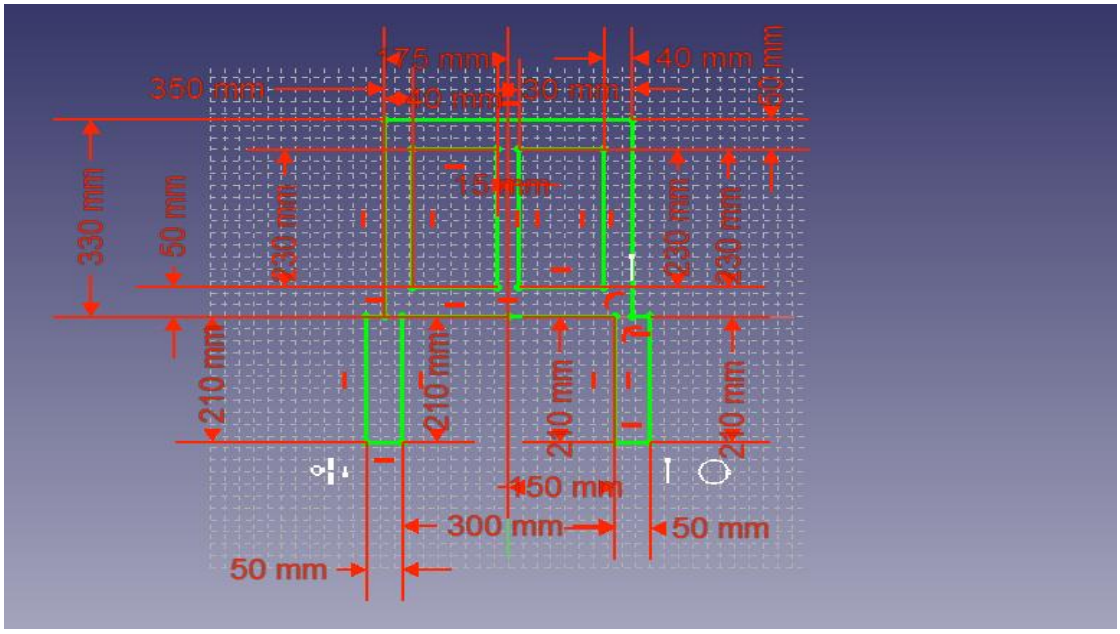
MODEL	JG-IA	CAPACITY	50/ML SCIENTIFIC RESEARCH

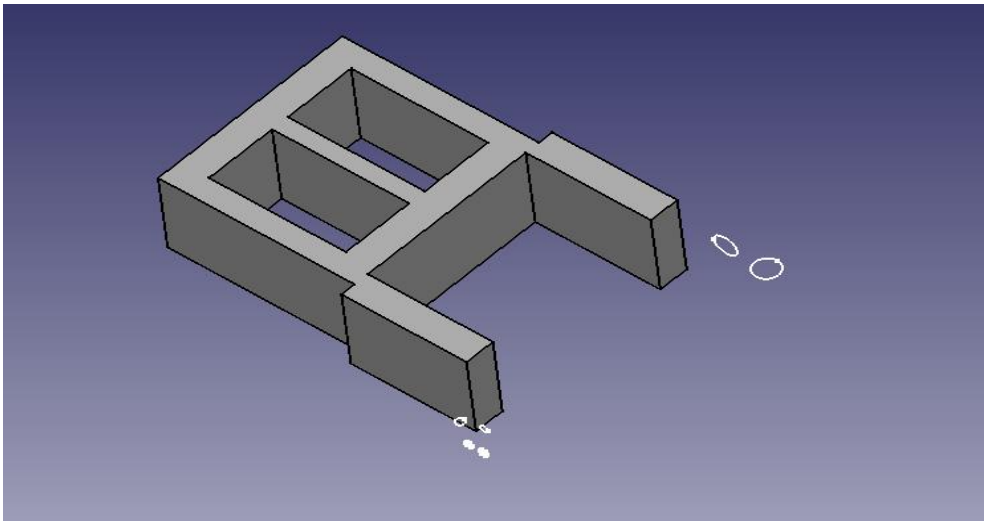
VOLTAGE	380 VAC	MAX OPERATING PRESSURE	256 MPA
PRESSURE DEVICE	HYDRAULIC SYSYTEM	MAX PRESSURE STROKE	170 MM
SAMPLE TUBE	Ø25 MM STAINLESS	PRESSURE PLATE SPEED	6.8 MM/S
DIMENSIONS	800×910×810	LARGEST VOLUME SAMPLE	50ML/TIMES

15.6.2 PART DIMENSIONS & DESIGN

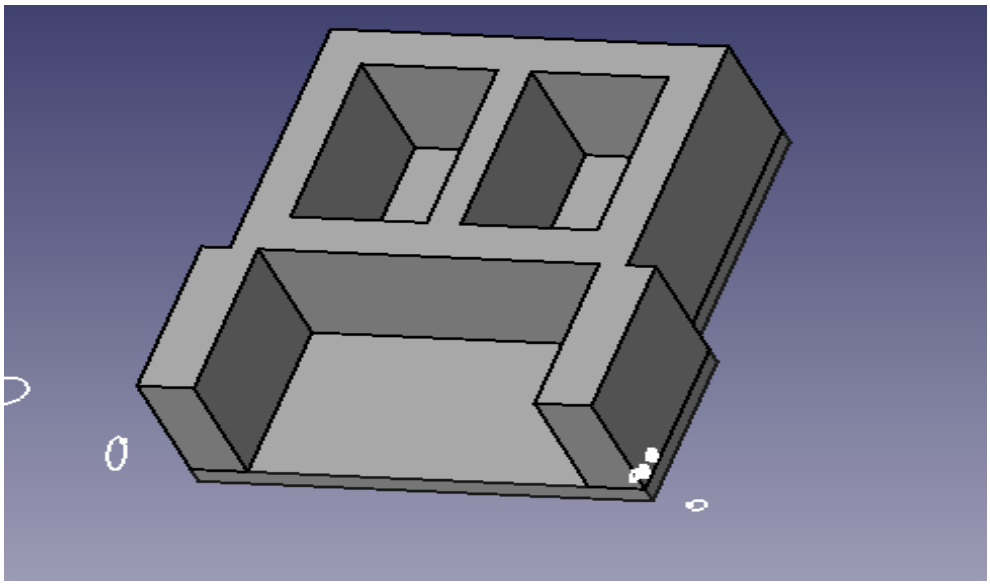
15.6.2.1 BASE PART

-box (sketch) with thickness =175 mm that contains the crankshaft and pistons to operate the process (breaking cells).



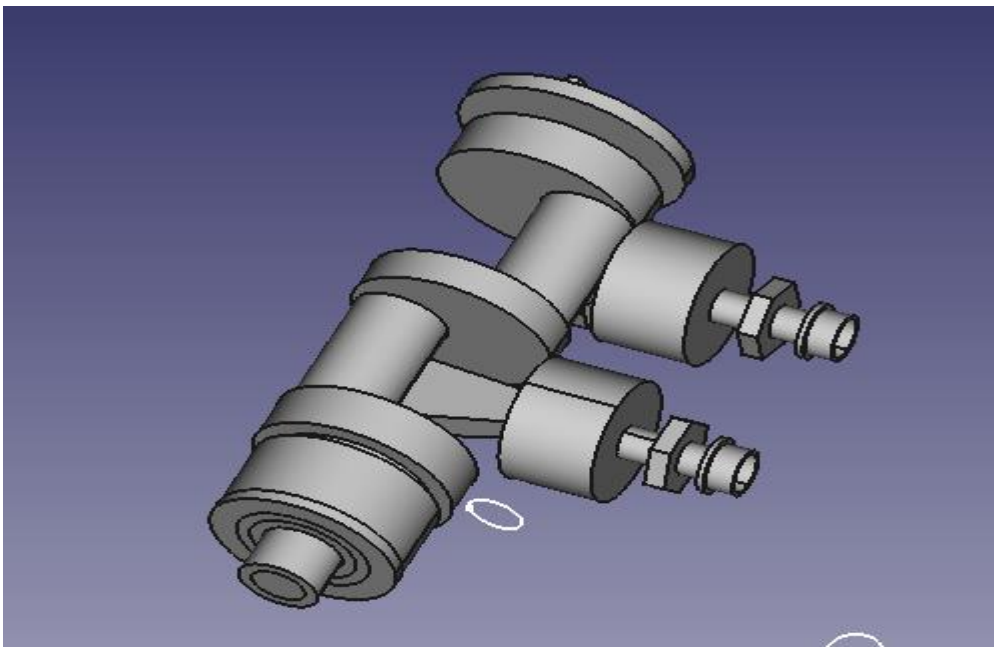


-Cover for Base with thickness =25mm

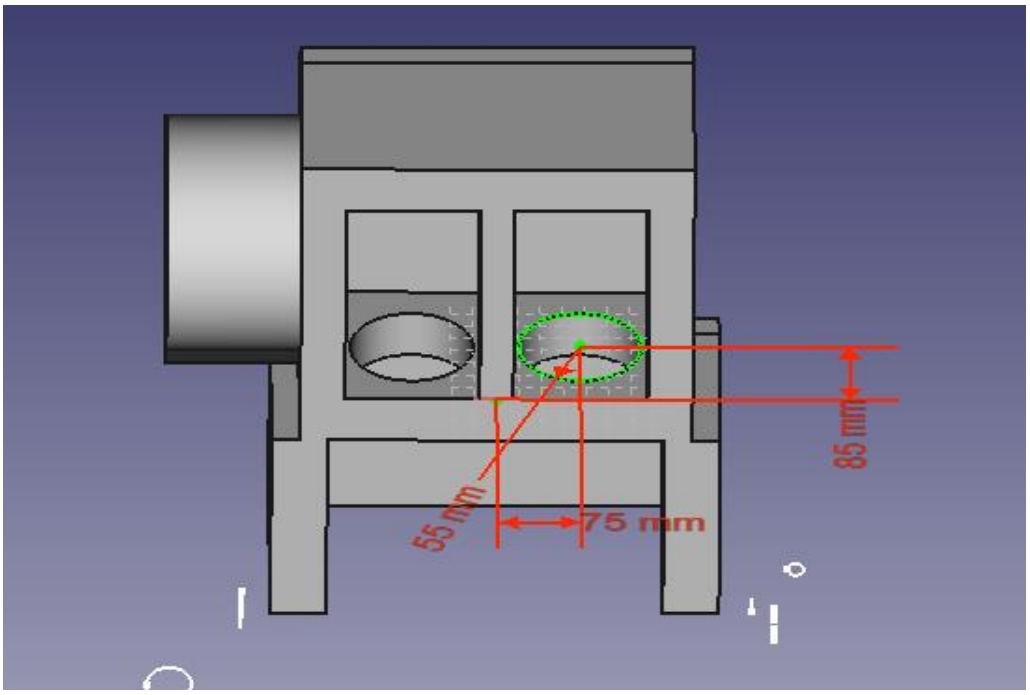


15.6.2.2 CRANK SHAFT AND PISTONS

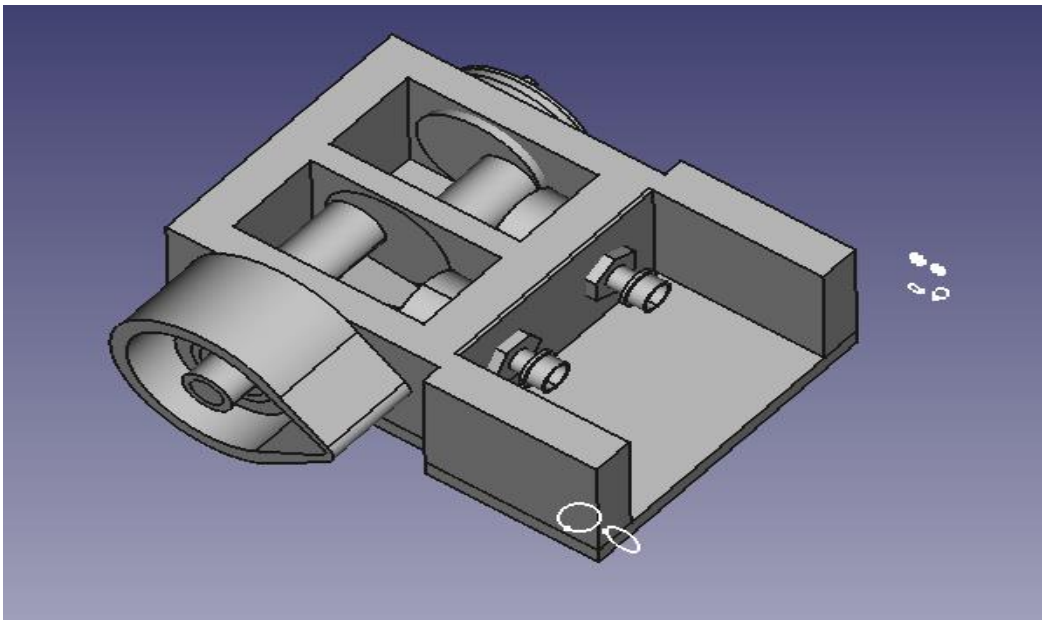
-Taking into account the dimensions of the shaft and pistons according to the box dimensions.



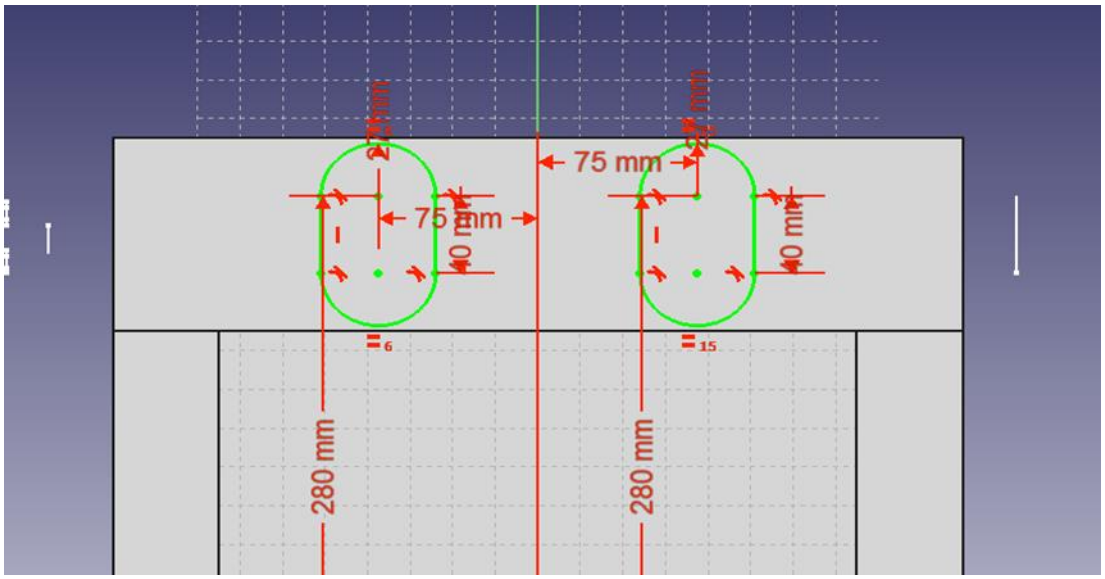
15.6.2.3 Pocket circle with R= 55mm to pass the cylinder of the pistons

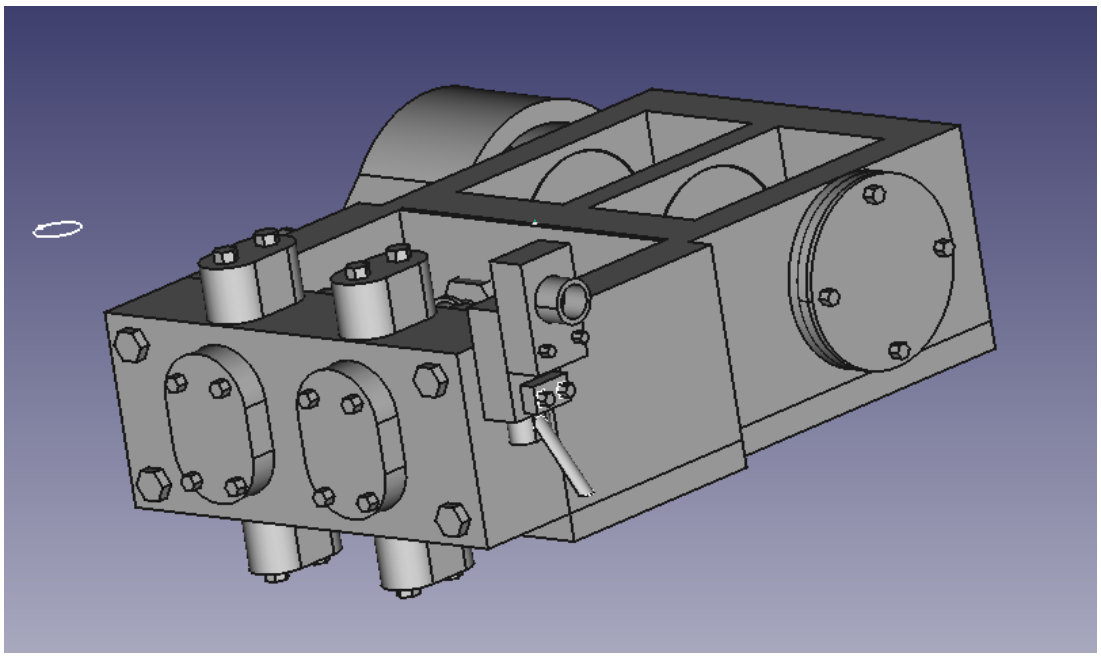


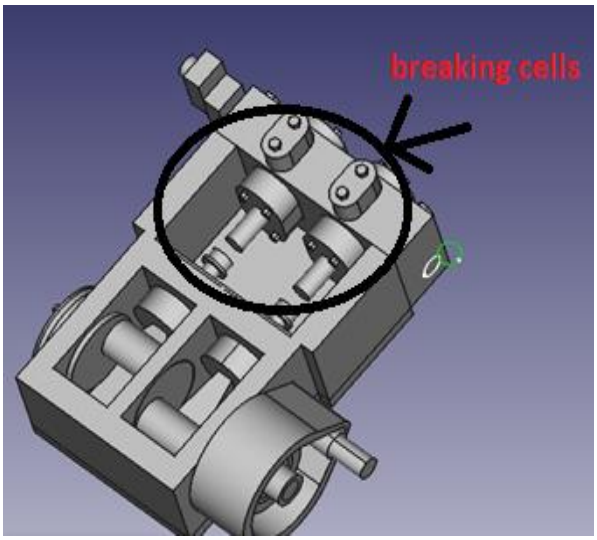
15.6.2.4 With same sketch for pistons and box bind the two parts together.

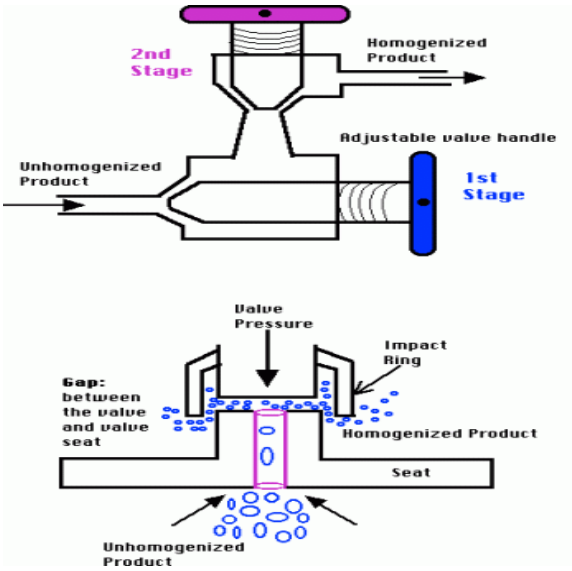


15.6.2.5 Draw the head of the machine where the cell will break due to the pistons and valve.

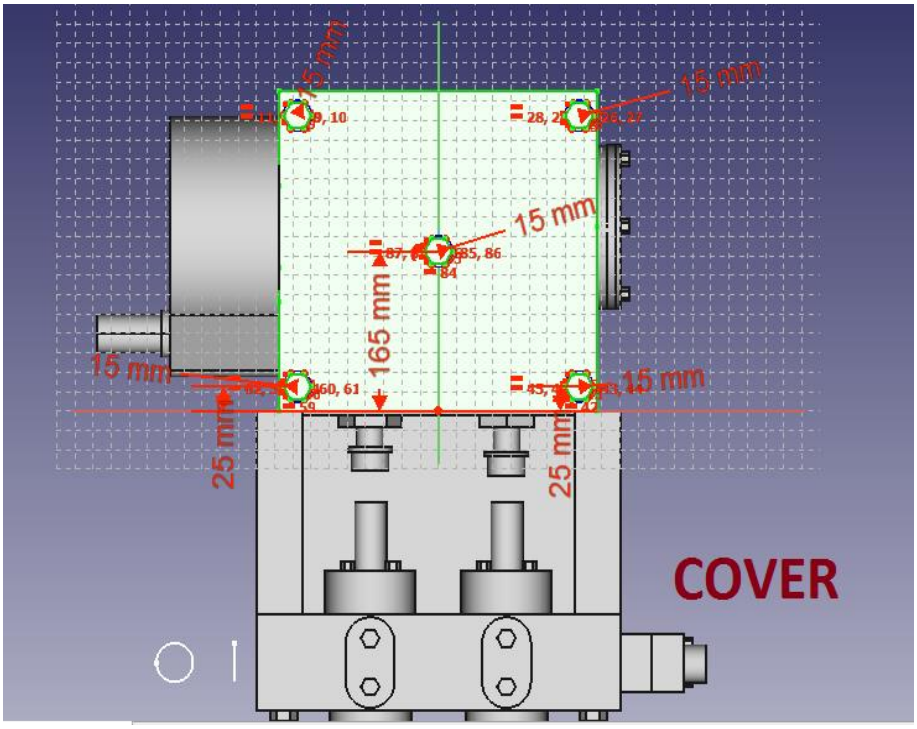


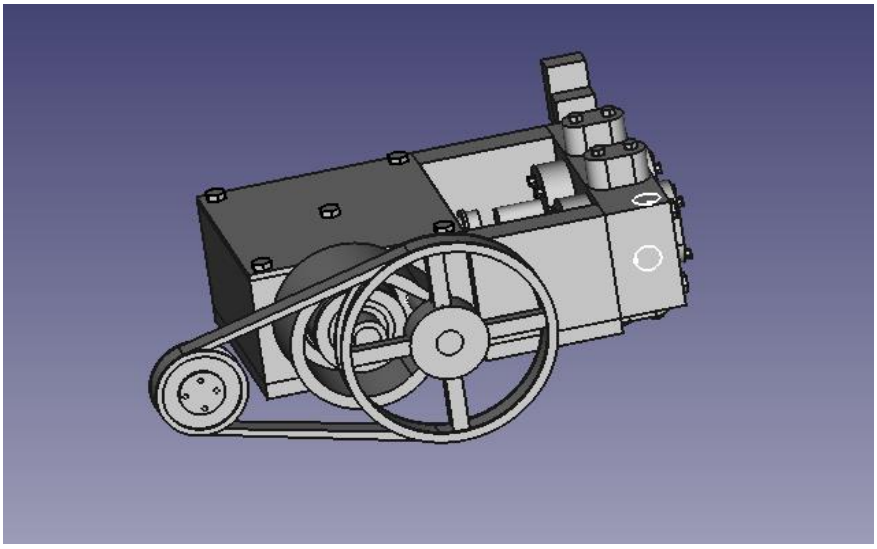






15.6.2.6 Putting the cover over the box and fixed it with bolts and added the shaft comes out from crank shaft and connecting to pulley, this pulley also connected to other one with belt.

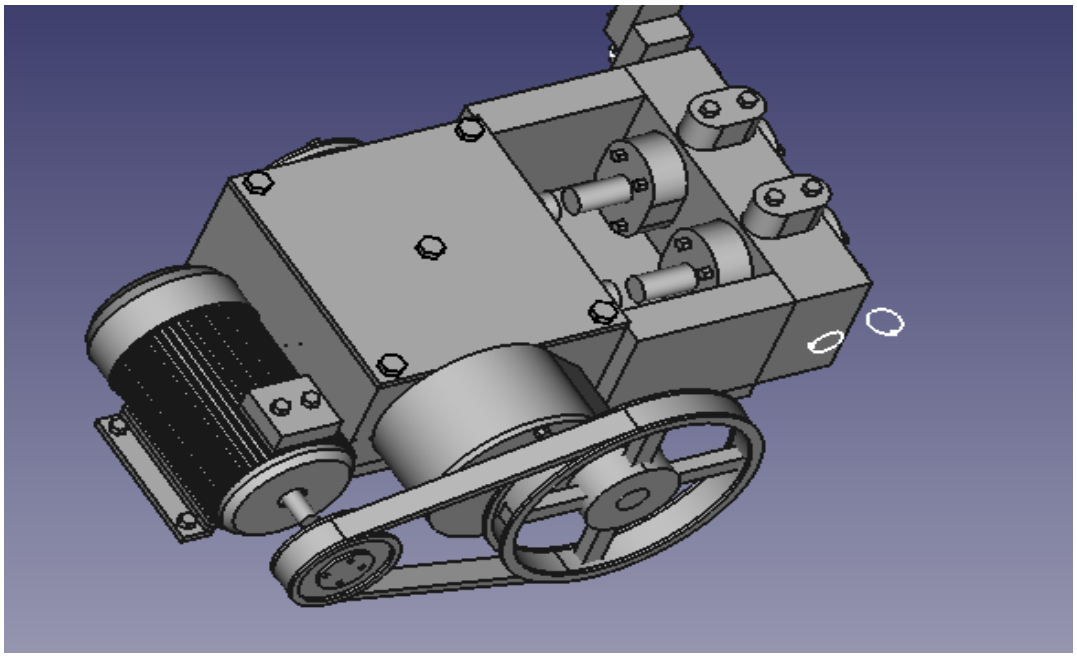




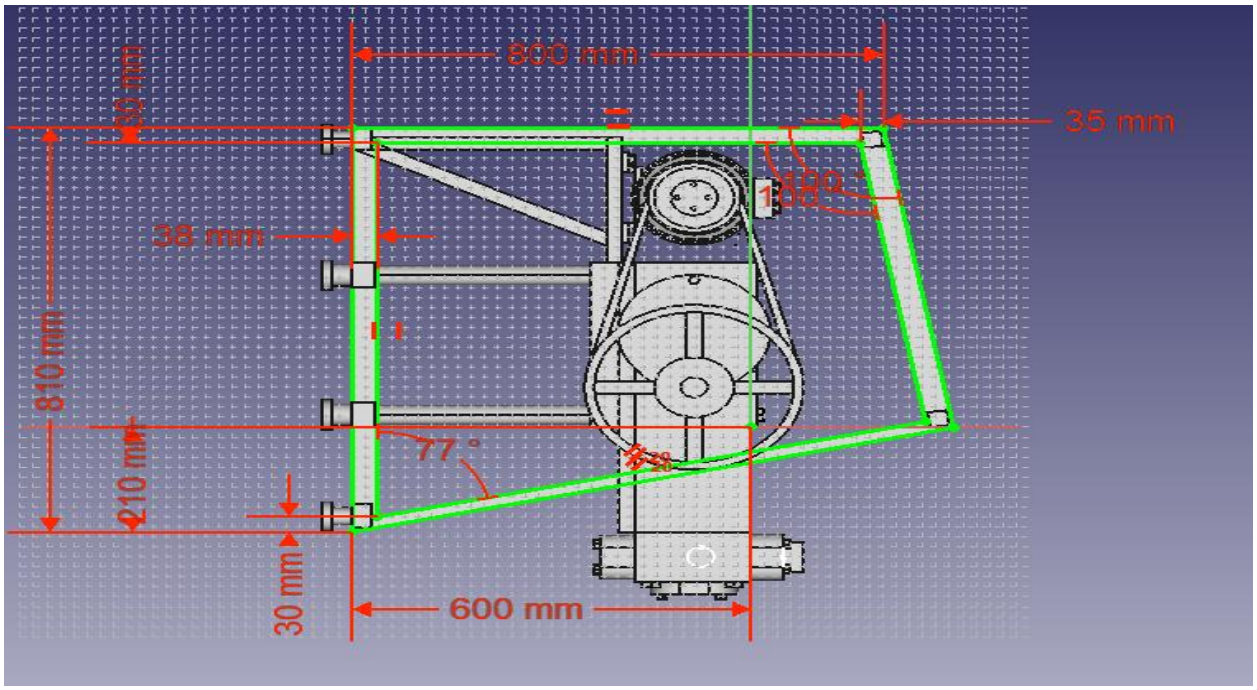
-belt thickness =10 mm

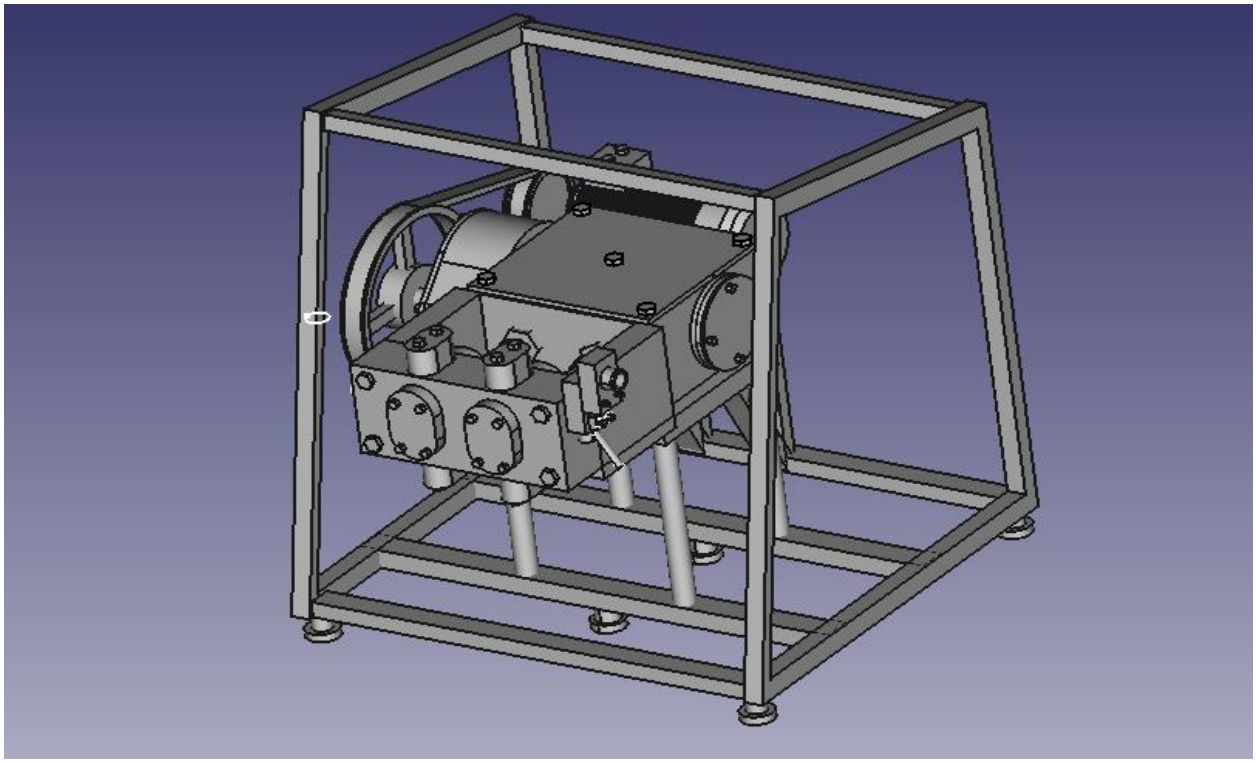
-Large circle with R=165 mm and small R=70 mm

15.6.2.7 Select a motor drive and connected to the small pulley to drive the second and the pistons start work

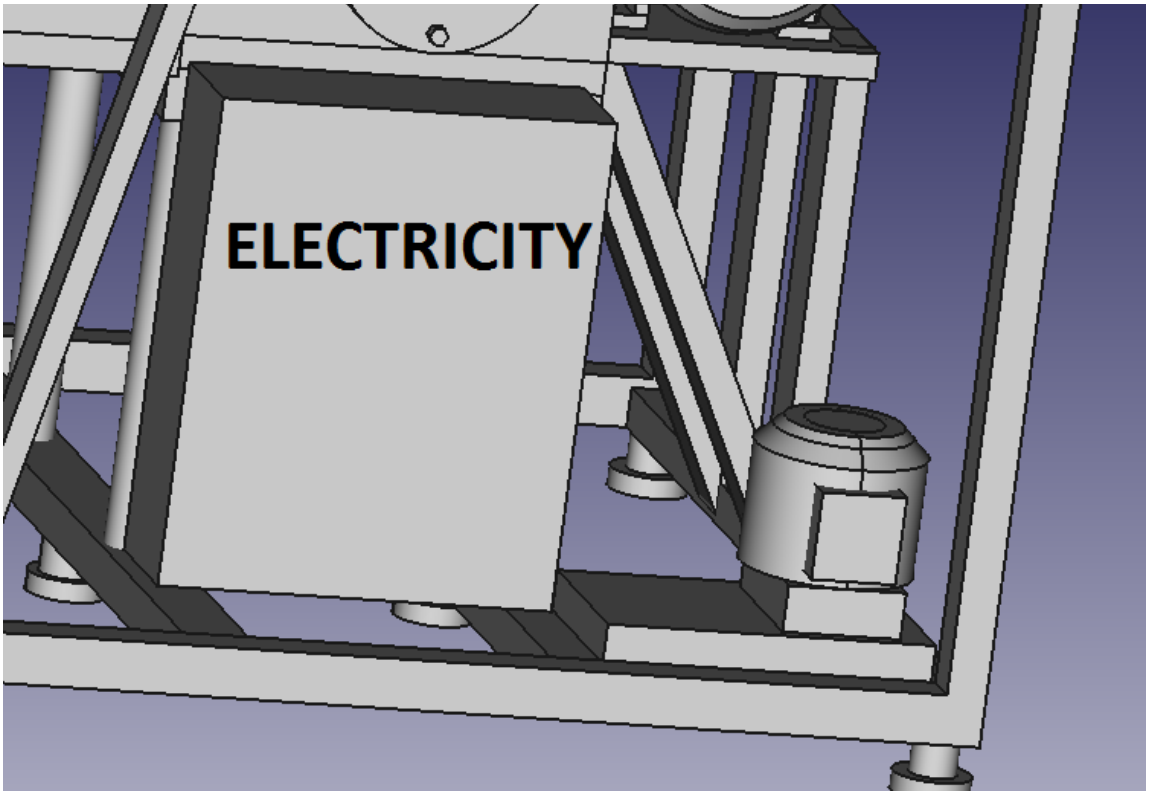


15.6.2.8 Drawing the boundaries of the machine and the basement for the box

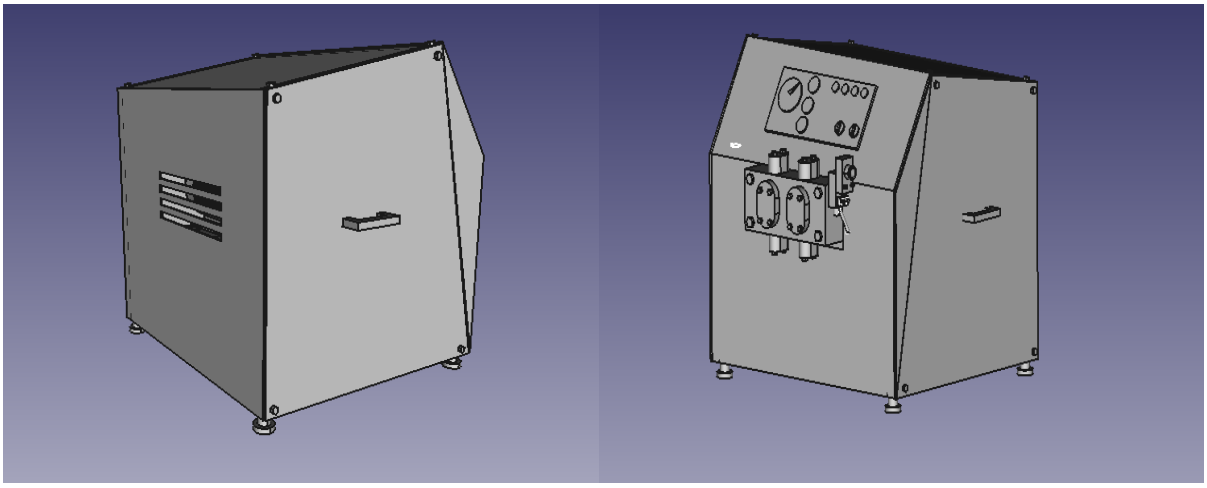




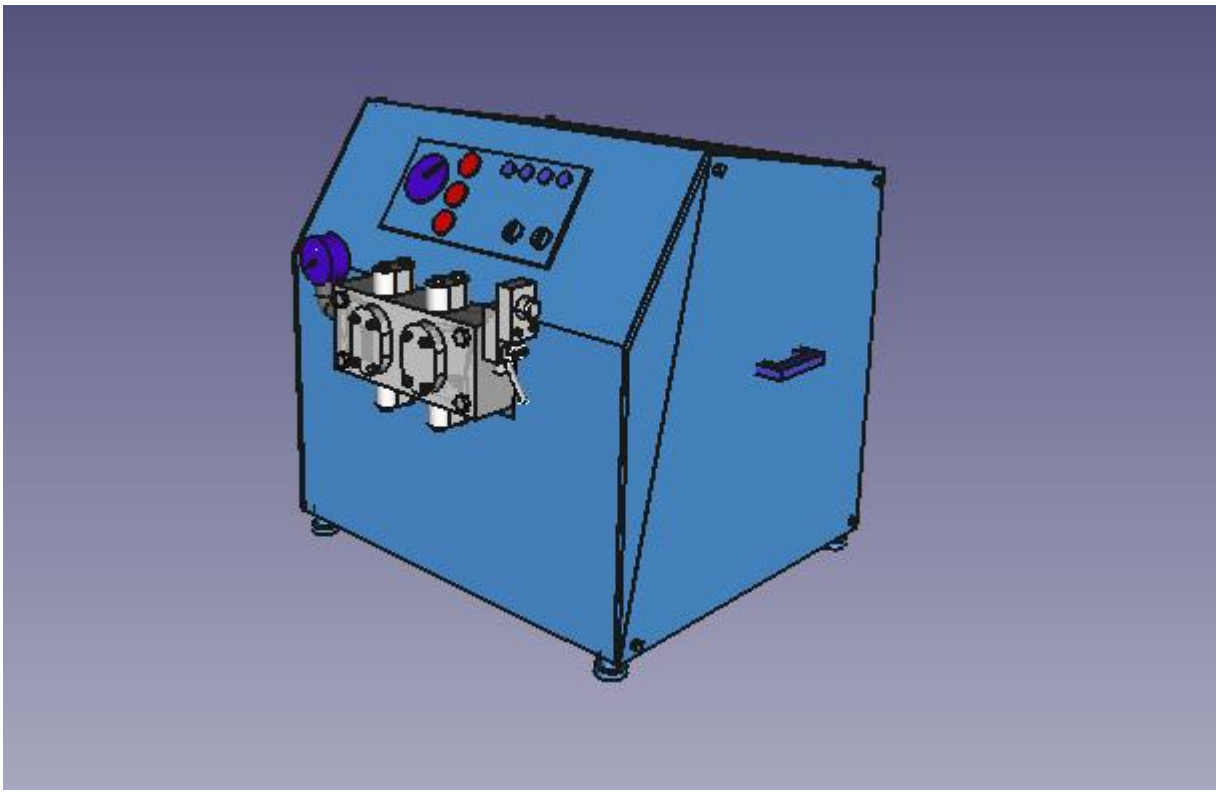
-Small motor and electricity box



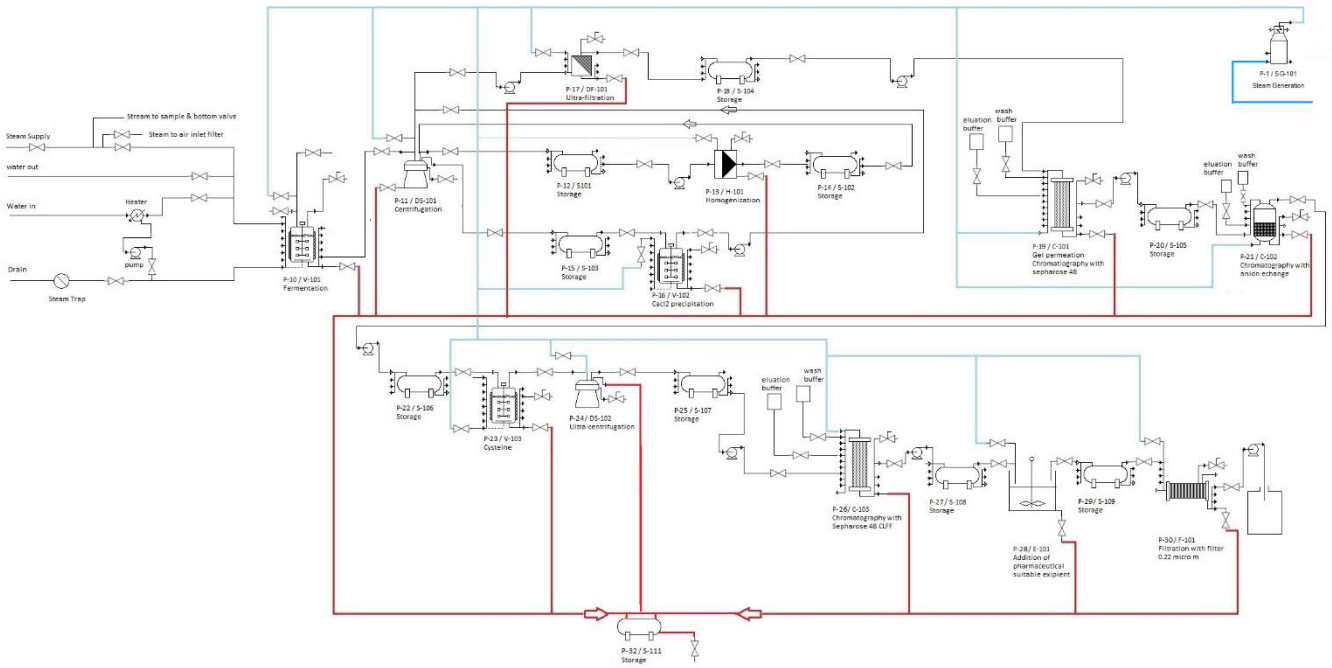
15.6.2.9 Finally close the machine with stainless wall



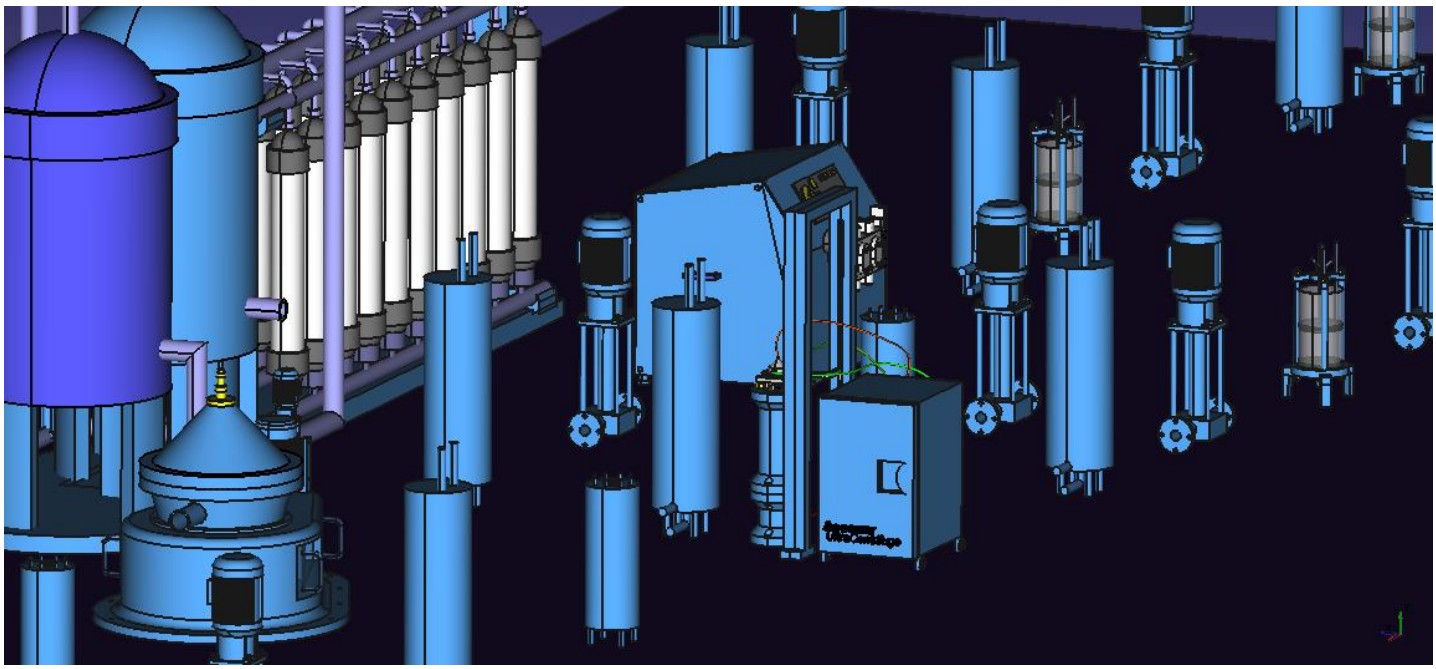
FINAL IMAGE: HOMOGENIZER

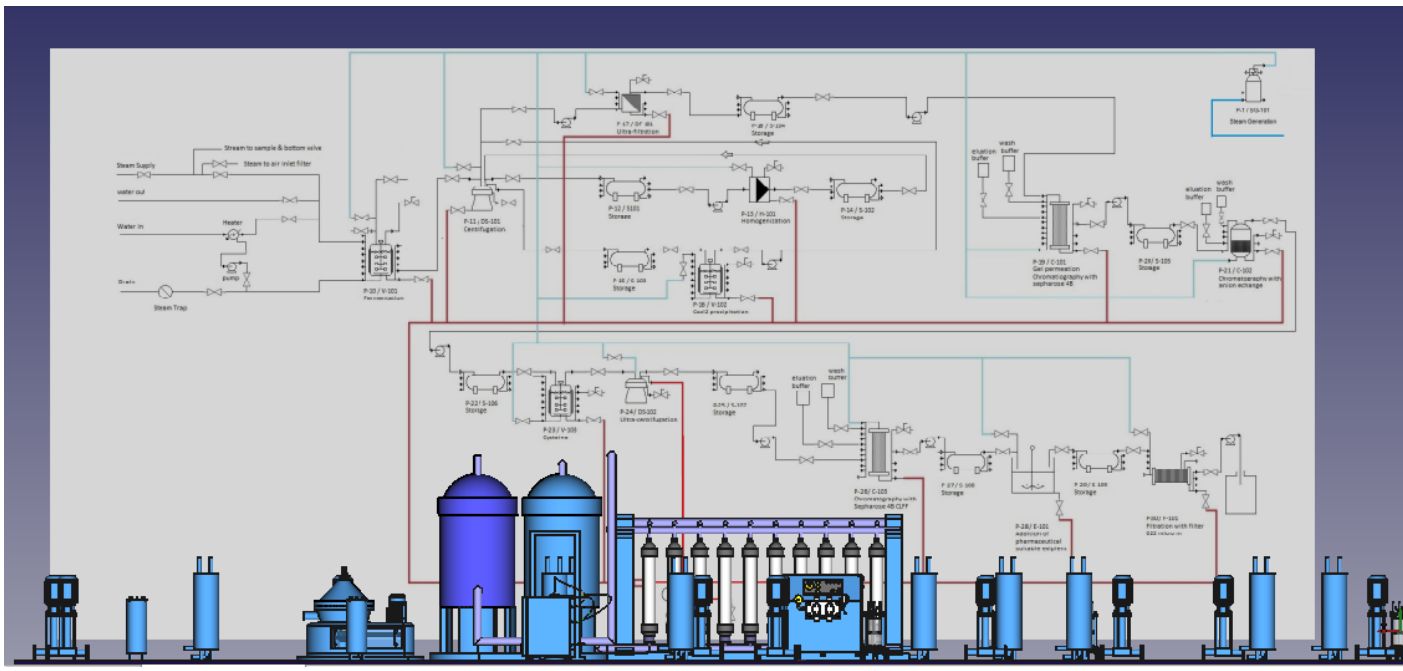


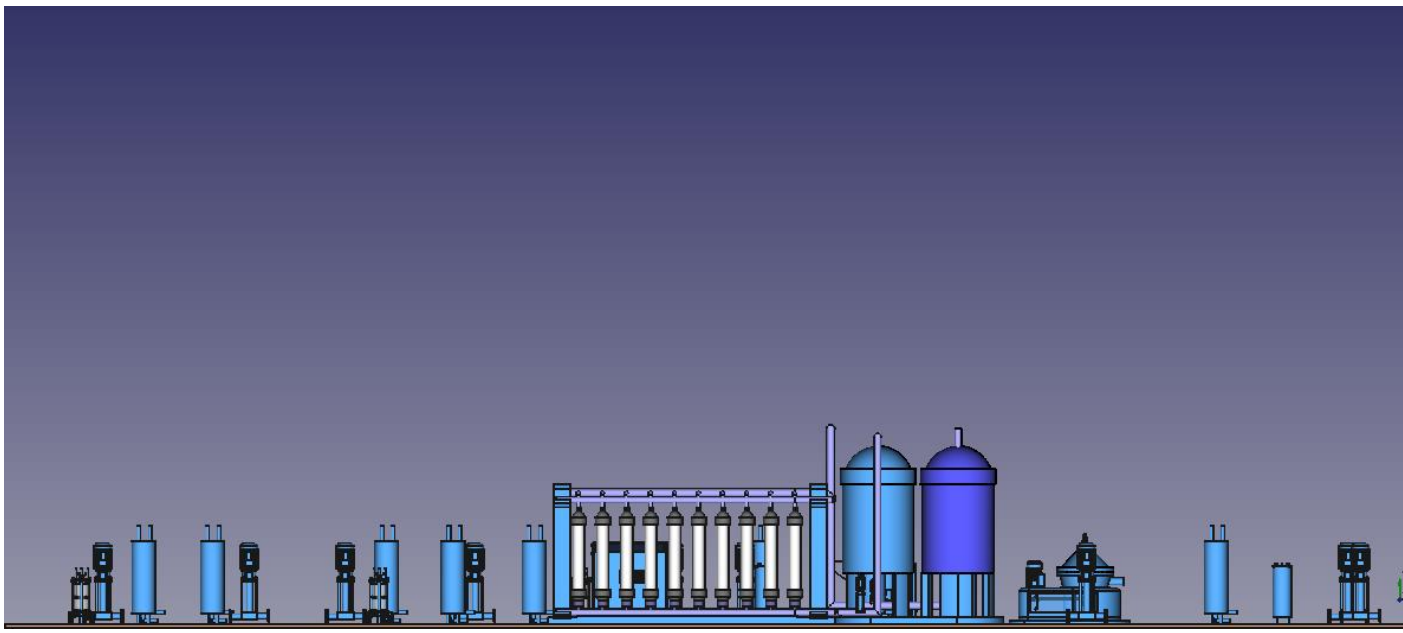
15.7 Final Assembly of the MEGBI Plant

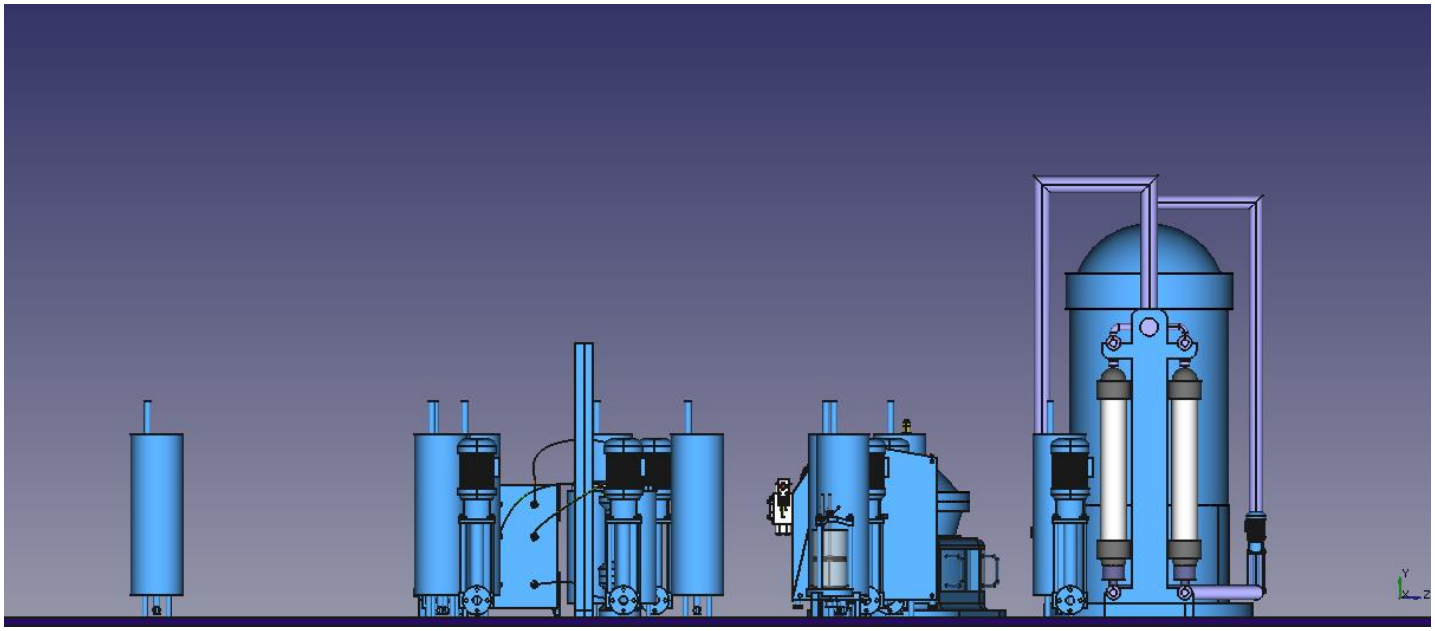


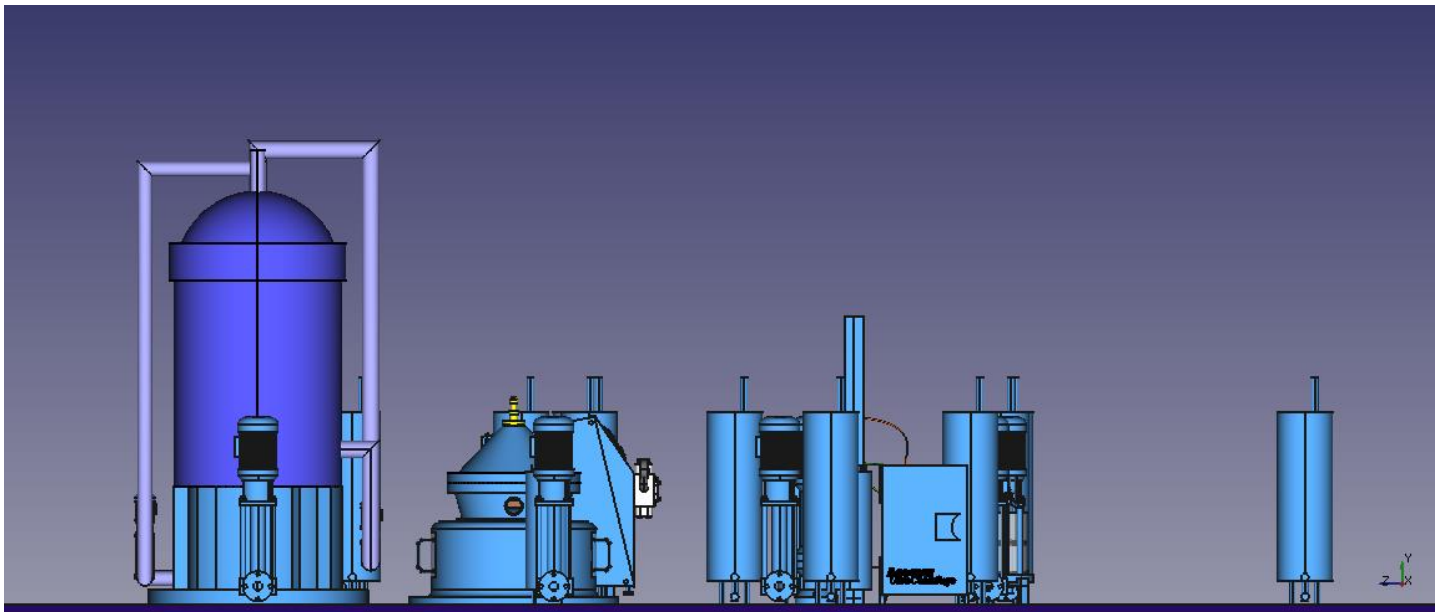
"Due to the lack of Assembly Features in FREECAD, Assembling These Parts Together. Was harder than Modelling Itself "– Jihad Samarji

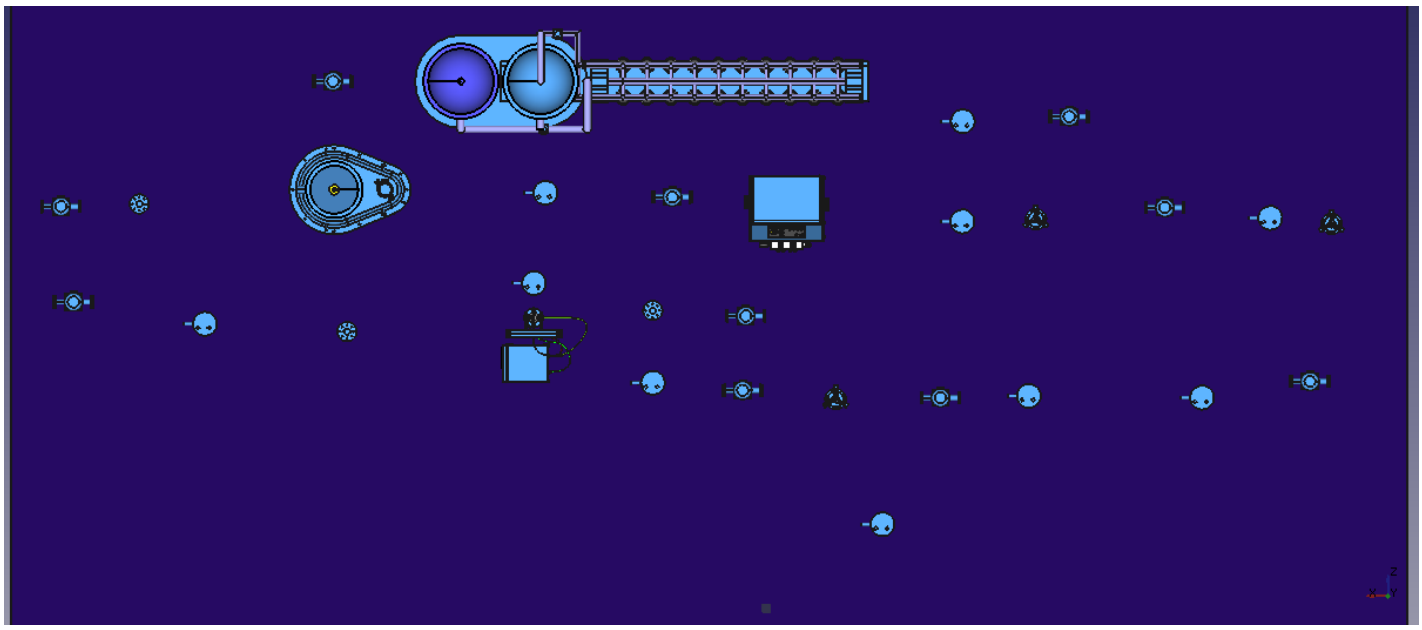


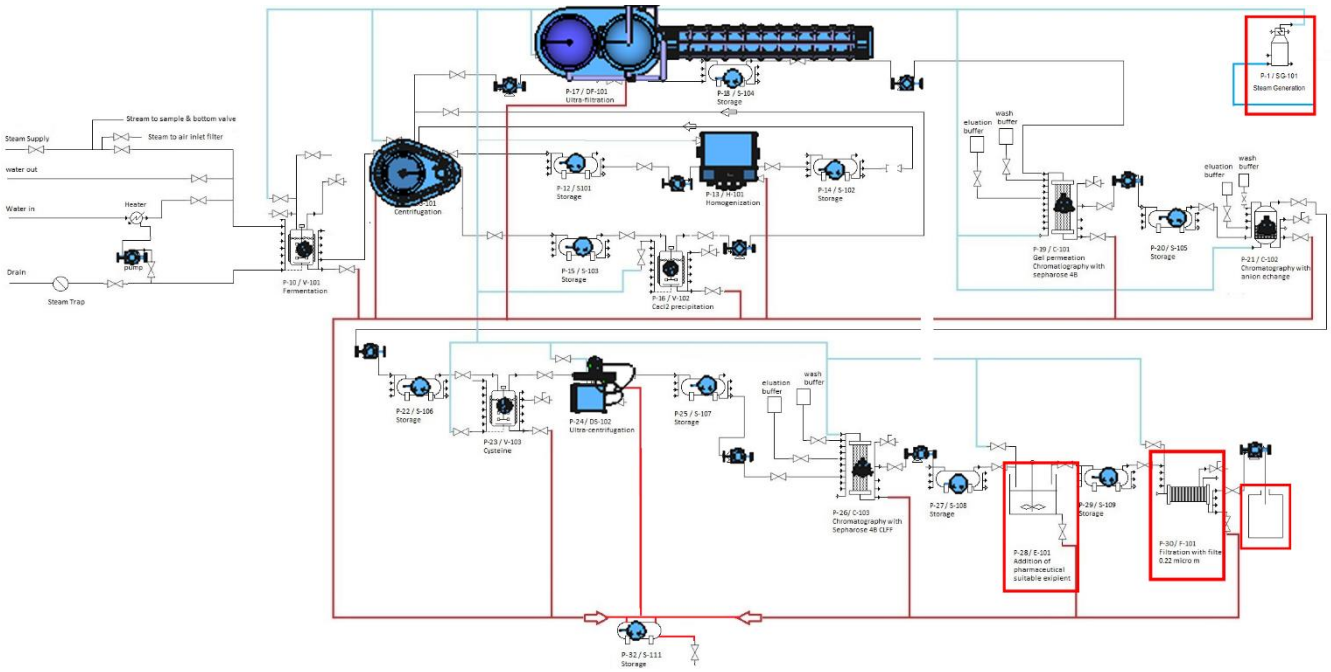


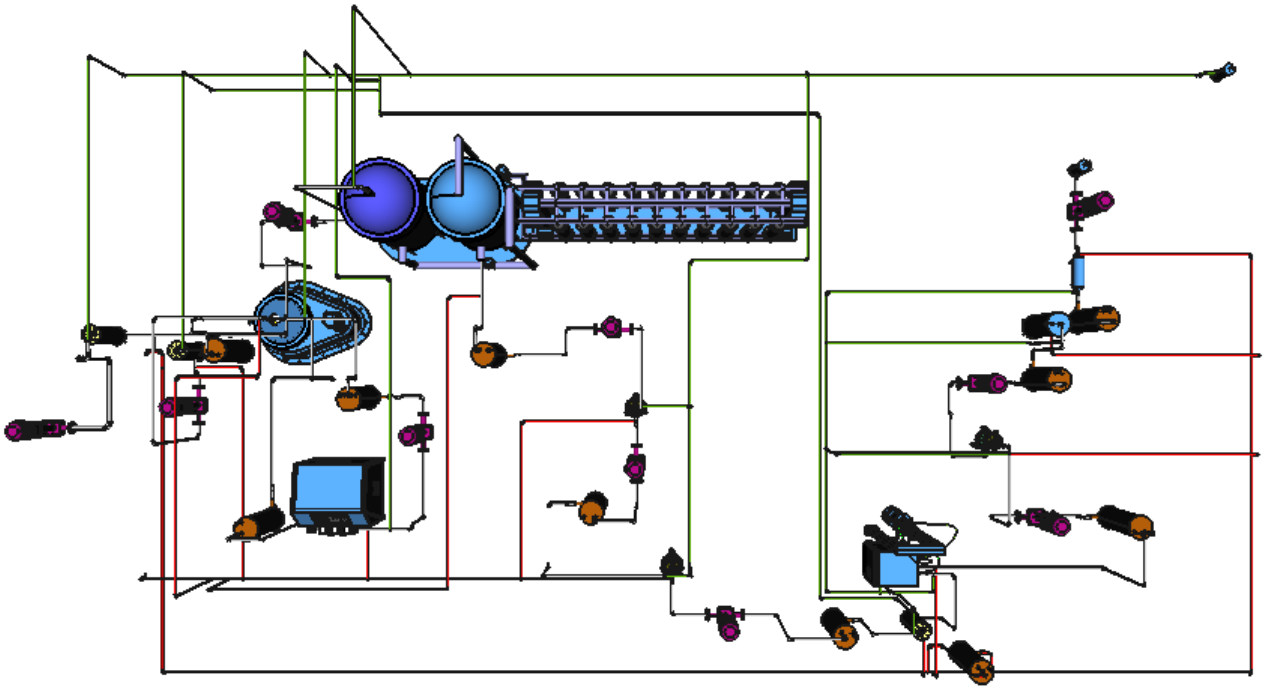


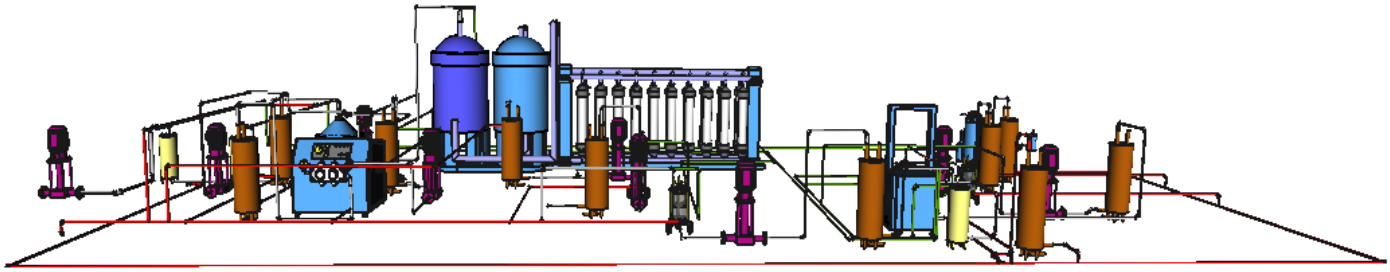







Stream to sample & bottom valve
Steam Supply
Steam to air inlet filter
water out
Water in
Heater
Drain
pump
Steam Trap
P-10 / V-101
Fermentation
P-11 / C-101
Centrifugation
P-17 / DF-101
Ultra-filtration
P-18 / S-104
Storage
P-12 / S101
Storage
P-13 / H-101
Homogenization
P-14 / S-102
Storage
P-15 / S-103
Storage
P-16 / V-102
CaCl2 precipitation
P-1 / SG-101
Steam Generation
elution buffer
wash buffer
P-39 / C-101
Gel permeation Chromatography with sepharose 4B
P-20 / S-105
Storage
P-21 / C-102
Chromatography with anion echange
P-22 / S-106
Storage
P-23 / V-103
Cysteine
P-24 / DS-102
Ultra-centrifugation
P-25 / S-107
Storage
P-26/ C-103
Chromatography with Sepharose 4B CLFF
P-27 / S-108
Storage
P-28 / E-101
Addition of pharmaceutical suitable exipient
P-29 / S-109
Storage
P-30 / F-101
Filtration with filter 0.22 micro m
P-32 / S-111
Storage





MODELLING AND ASSEMBLING MEGBI-VPP IS DONE

NEXT STEP: IS TO MAKE AN AUTOMATION SYSTEM TO SIMULATE THE WORK OF THIS PLANT

THIS CHAPTER CONTAINS INFORMATION GATHERED FROM PERSONAL EXPERIENCE AND THE INTERNET.

16 Automation System Design and GUI

This task was done as master thesis from Haitham Hindy, Lebanese University, Tripoli

Master Thesis

Automation of measurement of temperature, pressure and pH data and automation of fluid flow of a biotechnological production plant

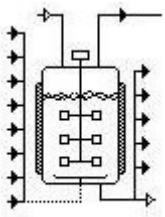
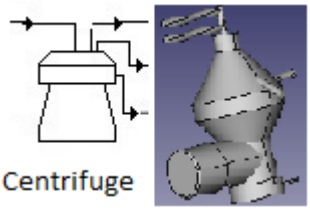
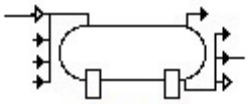
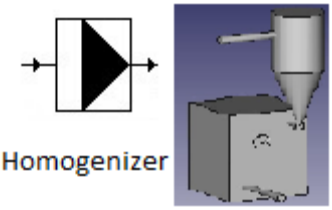
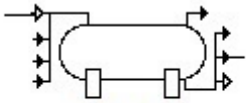
- Design of Software (State machines) for Homogenizer, Disc Stack Centrifuge in including CIP/SIP functional elements, Process Scale Gel Permeation and ion exchange chromatographic devices, Process Scale Ultrafiltration Device (6 weeks)
- Graphical User Interface for the automation of MEGBI-VPP downstream processing (DSP) unit (4 weeks)
- Adaptation of a Graphical User Interface to a Siemens S7 PLC system (6 weeks)
- Documentation (3 weeks)

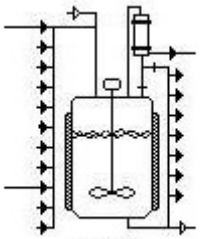
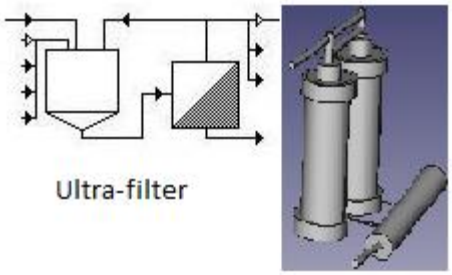
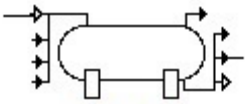
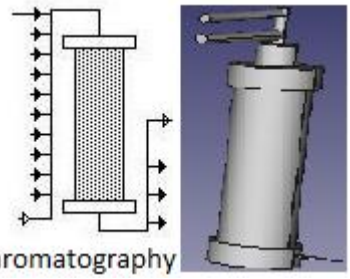
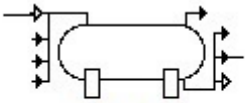
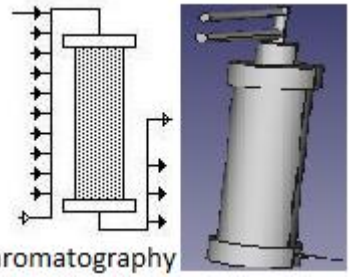
Keywords: measurement of temperature, pressure and pH data, Automation of fluid flow, PLC, Siemens S7, Programming, User Interface, C++/Java, Biotechnology

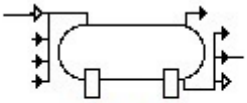
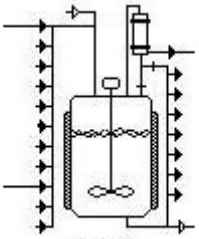
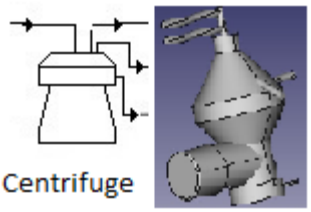
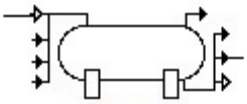
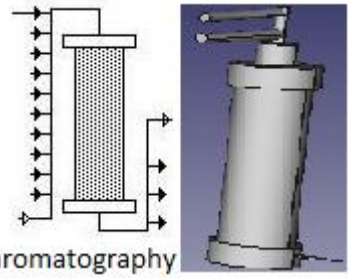
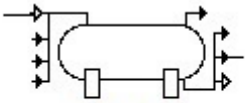
For automation system basics please refer to chapter 2, sections 15-17

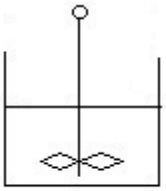
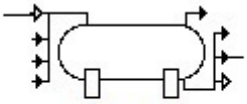
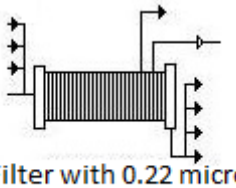
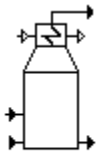
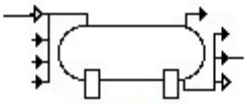
16.1 Automation System Specification

*Table 3: List of Process **devices** with machine, PI diagram symbol and instruments needed.*

PROCESS NUMBERS	DEVICES NUMBERS	PI DIAGRAM SYMBOL/CAD MODEL	VALVES / PUMPS	SENSORS
P-10	V-101	 Bioreactor	V1 V2 V3 V4 V5	T1 PH1 MF1 PR1 PO1
P-11	DS-101	 Centrifuge	V1 V2 V3 V4 V5 V6	T2 MF2 PR2
P-12	S-103	 Storage	V1	---
P-13	H-101	 Homogenizer	V1 V2 V3 PU1	T3 MF3 PR3
P-14	S-102	 Storage	V1	---
P-16	V-102		V1 V2 V3 V4 V5	T4 MF4 PR4

		 Precipitation		
P-16	DF-101	 Ultra-filter	V1 V2 V3 V4 V5 PU2	T5 MF5 PR5
P-17	S-103	 Storage	V1	
P-18	C-101	 Chromatography	V1 V2 V3 V4 V5 V6 PU3	T6 MF6 PR6
P-19	S-104	 Storage	V1 PU4	
P-20	C-102	 Chromatography	V1 V2 V3 V4 V5 V6	T7 MF7 PR7

P-21	S-105	 Storage	V1 PU4	
P-22	V-103	 Precipitation	V1 V2 V3 V4 V5	T8 MF8 PR8
P-23	DS-102	 Centrifuge	V1 V2 V3 V4	T9 MF9 PR9
P-24	S-106	 Storage	V1	
P-25	C-103	 Chromatography	V1 V2 V3 V4 V5 V6 PU5	T10 MF10 PR10
P-26	S-107	 Storage	V1	

P-27	E-101	 Blender	V1 V2 V3 V4	
P-28	S-108	 Storage	V1	
P-29	F-101	 Filter with 0.22 micro m	V1 V2 V3 V4 V5	T11 PR11 MF11
P-30	S-110	 Steam generater	V1	P1
P-31	S-109	 Storage	V1	---

With this list can make the screen, we have painted a miniature in a paper A4 link the instruments together in order to process the next process and we have introduced the valves to get this:

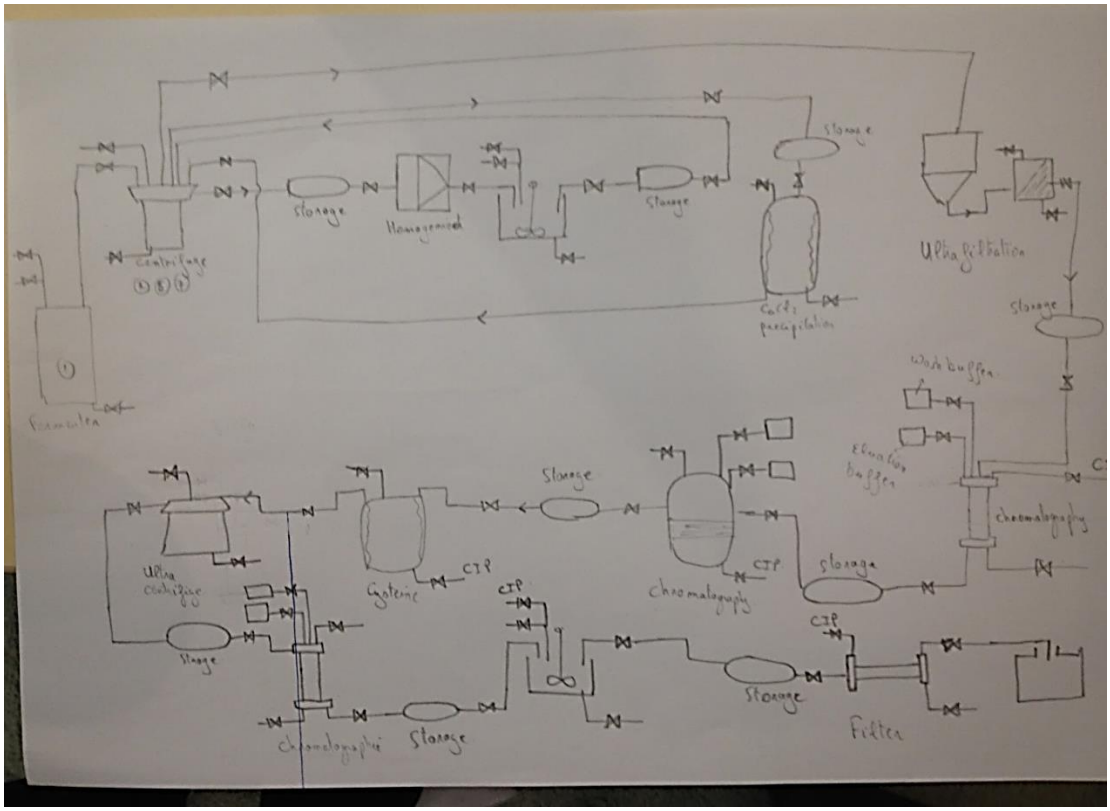
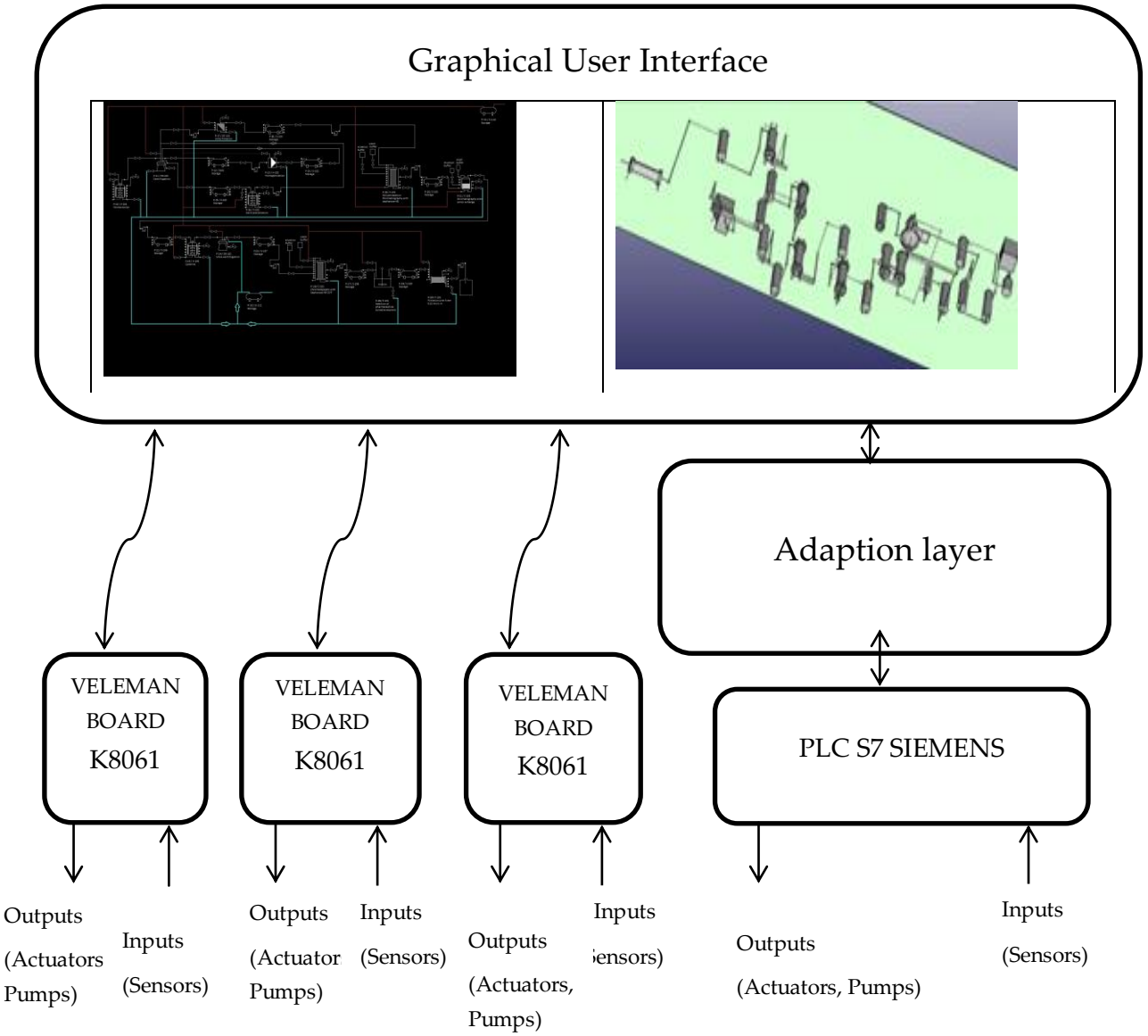


Figure 22: *Painting the Downstream Processing.*

Next step we have used this image to create the graphical user interface, we have collected the SuperPro with paint to make all instruments and process together and get the image:


Graphical User Interface
Adaption layer
VELEMAN BOARD K8061
VELEMAN BOARD K8061
VELEMAN BOARD K8061
PLC S7 SIEMENS
Outputs (Actuators Pumps)
Inputs (Sensors)
Outputs (Actuator Pumps)
Inputs (Sensors)
Outputs (Actuators, Pumps)
Inputs ensors)
Outputs (Actuators, Pumps)
Inputs (Sensors)

16.2 Graphical User Interface

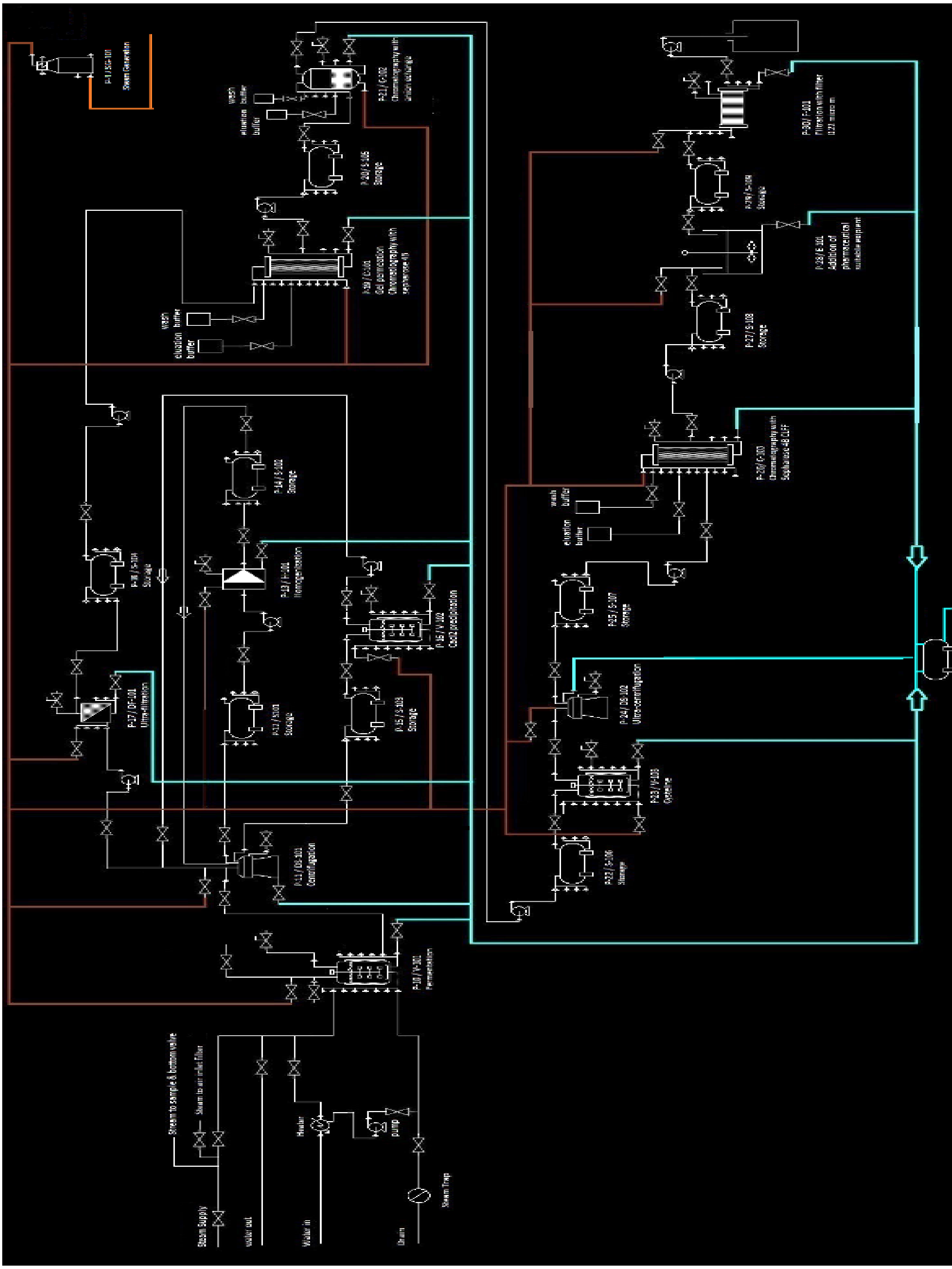


Figure 2.23: *PI Diagram which represents the MEGBI-VPP Processing.*

So by using the concepts and the specification of the production for the vaccine, we have organized the PI diagram for the MEGBI-VPP Processing and finally we get the PI diagram full needed to continue our project and to be the first step that has done and now this PI diagram (figure 2.1) represents the MEGBI-VPP Processing. On this PI diagram we have putted 3 pipelines white represents the road which will pass by the vaccine and some materials adding in many machines, we thought about cleaning the machines, everything need clean after use to get a perfect production, for that we have created the 2 next pipelines red and blue, the red one is the road of the steam water coming from a steam generator and the other one is the road of the dirty water ,it's the dirt coming from the production of the vaccine hepatitis b and beside the bioreactor we have added the temperature control system specific for it ,we have putted the valves necessary on the pipes to controls the in and out of fluid for the machine , and we have putted the pumps but we make look about the position that we have to use pump , so as we know if there is a elevation between the machine we need a pump so that is the condition that we have based on it.

Next have to use the PI diagram on Python to create the control screen, we have passed by many problems on python start by how to install, how to run, and how to use, we have searched on internet sites to answer on this questions, and we get the answer needed by the Python book[49] and internet sites[6] then we have used this reference for knowing the codes needed on Python to create this screen and how we should use it, first the idea of the control screen is controlling the fluid flow by open and close the valves, turning on and off the pumps, watching the status of each instruments on this screen for that we have needed buttons, labels texts list box background and window…

The hard job was on installing the Python, run it and how to choose the specific code for each thing we have to create on the screen so we have putted a plan about the things that we will show on the control screen, so as we talk before we need buttons by a specific code on Python we have created 68 buttons, 58 for valves and 10 for pumps , and we have

[49]Book name: Wx Python in Action

organized it like each valve beside it the button specific for it , so we have identified the position of the valve to know where we will put this button and we did this for the 68 buttons. Before button we have used the PI diagram created with a specific code on Python to the background of the control screen and with a specific code too we have created a window for this screen and we have created a bar status for this screen with 2 buttons 'exit' 'about', plus we have written on 'about' information about this screen and what it's represents. Next we have created two list box (one for valves, other for pumps) concerning the list name of the actuators (valve or pump) beside it we have putted a label for each actuators to shows on it the status of the actuators, this status we will use it to controls the actuators by open/close or turn on/off by a 'ON' or 'OFF' on label, so we have created on Python a condition between the label and button which we can use the click on button to get 'ON' for first click 'OFF' for other for a manual work, and for the full automation we have putted a "Turn ON" button that we will click on it to begin the production process. Now we have the window with the PI diagram background, buttons, and two list box and if we click on button we get the status of it on label in list box this for a manual work and we have the "Turn On" button, So the control screen is done and ready to use (figure2.2). About all specific codes used you can see the full program in Annex2 and see the codes.

Some difficulties during installation

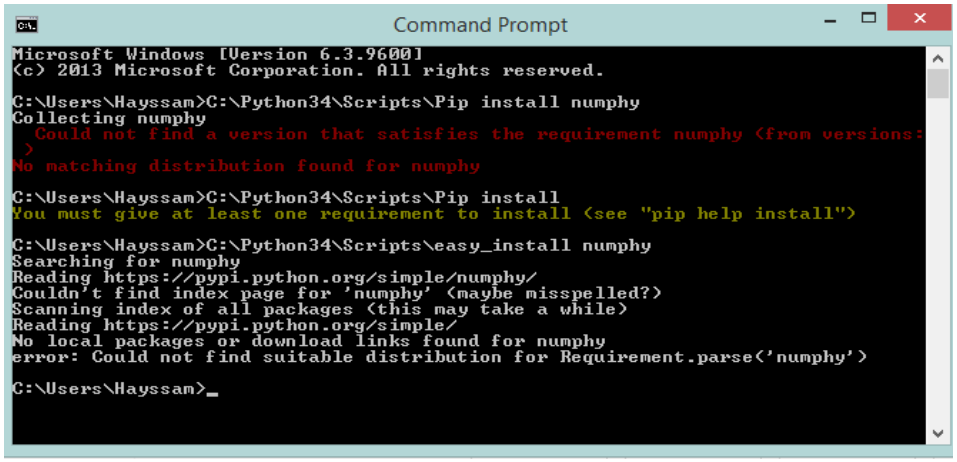


Figure 24 : **Error Command 2**

To repair this error, must upgrade the pip package:

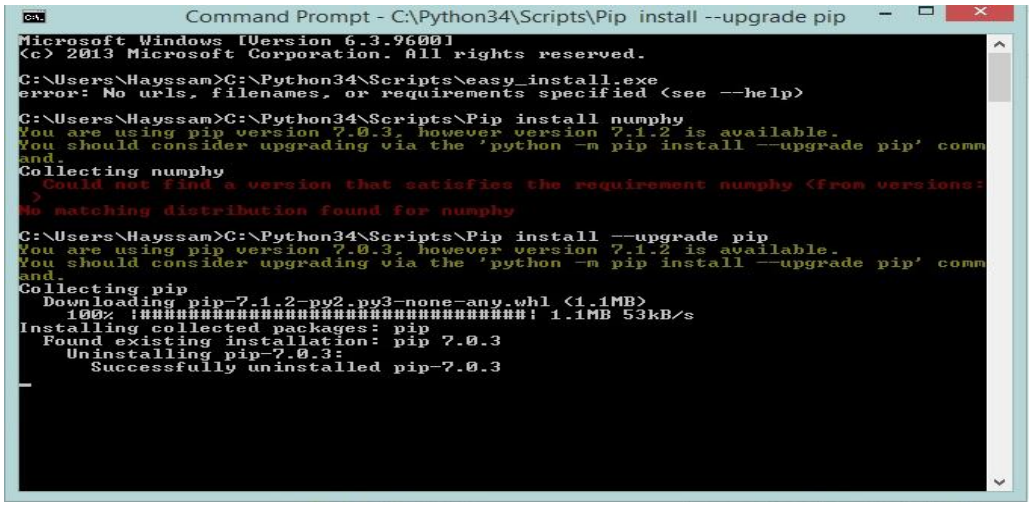


Figure 25: ***First step of upgrading.***

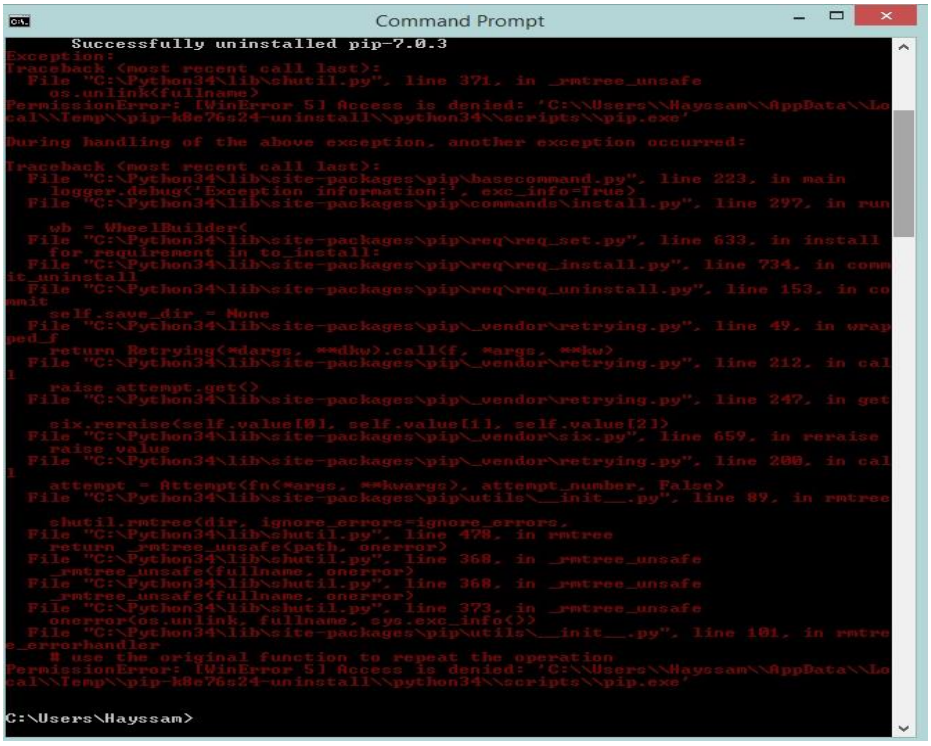


Figure 26 : *Done step of upgrading.*

For complete the installing must define the entire package like that example and run it:

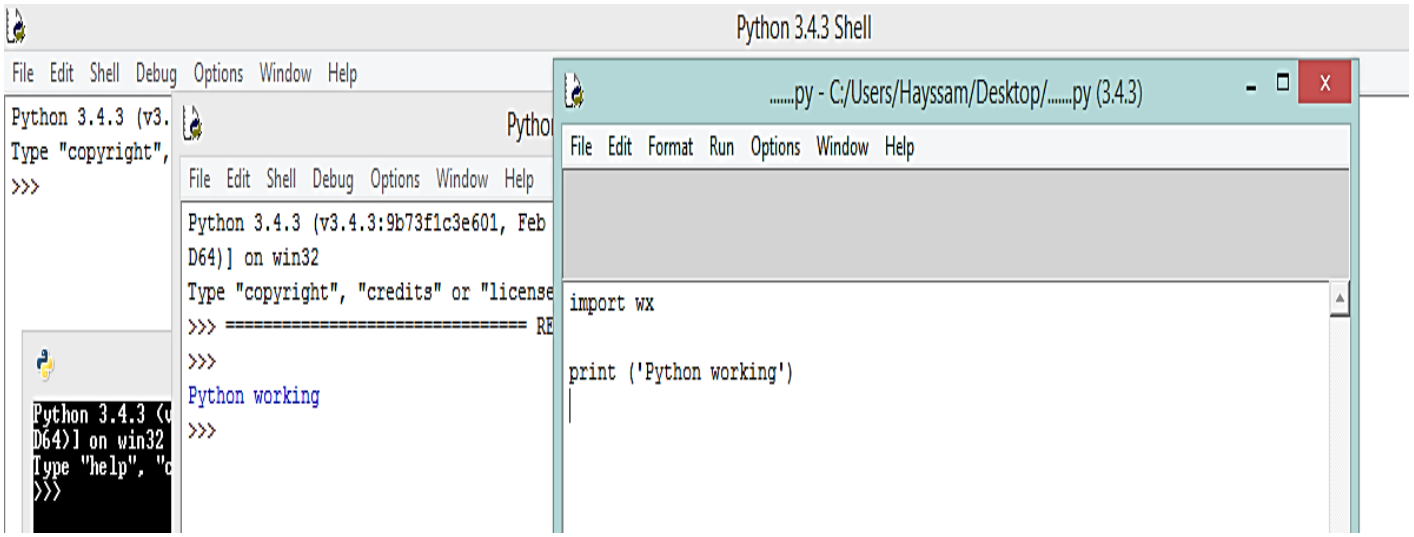


Figure 27 : *Python working.*

After installing and running the Python, must continue to written:

First how to insert a background, that background should be the screen, the previous image was painted must be that background, the Python had a code to use an image like a background we used the code to my image.

This is the code:

```
#**********************                    panel frame                      **********************
#*************************************************************************************************

BACKGROUND_IMAGENAME =\"C:\Users\Hayssam\Desktop\GUI 220415MEGPI-VPP_PI_DIAGRAM.PNG"

class MyBackgroundPanel(wx.Panel):

    def __init__(self, parent):
        wx.Panel.__init__(self, parent)
        self.bmp = wx.Bitmap(BACKGROUND_IMAGENAME)
        self.SetSize(self.bmp.GetSize())
        self.Bind(wx.EVT_PAINT, self.on_paint)


    def on_paint(self, event = None):
        dc = wx.BufferedPaintDC(self, self.bmp)
```

Figure 28 : *Python 1*

After this we had created a window from a code across written a new class, use the wx.Frame function, put name for this window and some developed.This is the code:

```
class MyFrame(wx.Frame):

    def __init__(self, parent = None, title = "MEGBI Vaccine Pilot Plant (MEGBI-VPP) Overview Upstream & Downstream Process"):


        wx.Frame.__init__(self, parent, -1, title)

        panel = MyBackgroundPanel(self)

        LABELSTYLE = wx.BORDER_SUNKEN | wx.ST_NO_AUTORESIZE | wx.ALIGN_CENTER_HORIZONTAL


        menuFile = wx.Menu()
        menuFile.Append(1, "&About...")
        menuFile.AppendSeparator()
        menuFile.Append(2, "Exit")
        menuBar = wx.MenuBar()
        menuBar.Append(menuFile, "File")
        self.SetMenuBar(menuBar)
        self.CreateStatusBar()
        self.SetStatusText("Welcome to MEGPI Project!")
        self.Bind(wx.EVT_MENU, self.OnAbout, id=1)
        self.Bind(wx.EVT_MENU, self.OnQuit, id=2)


    def OnAbout(self, event):
        wx.MessageBox("This is a Screen controller of MEGPI Project","Welcome to my python", wx.OK | wx.ICON_INFORMATION, self)
    def OnQuit(self, event):
        self.Close()
```

Figure 29 : *Python 2*

After the frame class, must put the finale class for the main loop, this is for show the background and the frame without it nothing done.

This is the code:

```
#**********************                main definition and loop              ********************
#*************************************************************************************************

def main():
    """Testing"""
    MyBackgroundPanel = wx.PySimpleApp(wx.Panel)
    f = MyFrame()
    d=ValveListBox()
    d.Show()
    f.Center()
    f.Show()
    MyBackgroundPanel.MainLoop()

if __name__ == "__main__":

    main()
```

Figure 30 : *Python 3*

Next increased buttons for valves and pumps cause needed to click on it to open and close the valve, turn on or to turn off the pump, for that we used the code in python to create

the buttons but we must specify the position of the valve or pump to put the button near of it, to know that button to that valve or pump.

The code in python given much of details like we can name the button and position for it in screen so that easy to create in my screen . I have 67 valves and 10 pumps.

This is the code:

```
            # Create Valves

               #V1

wx.StaticText(panel,-1," V1 ",(18,300))


self.button_V_1 = wx.Button(panel, -1, "V1", pos=(95,100),size=(20,20))
self.Bind(wx.EVT_BUTTON, self.V1, self.button_V_1)


def V1(self, event):
        wx.MessageBox("V1 is Open ","Open", wx.OK|wx.ICON_INFORMATION)
```

Figure 31 : *Python 4*

Getting this screen in first try, if we click on V1 will be given "V1 is Open":

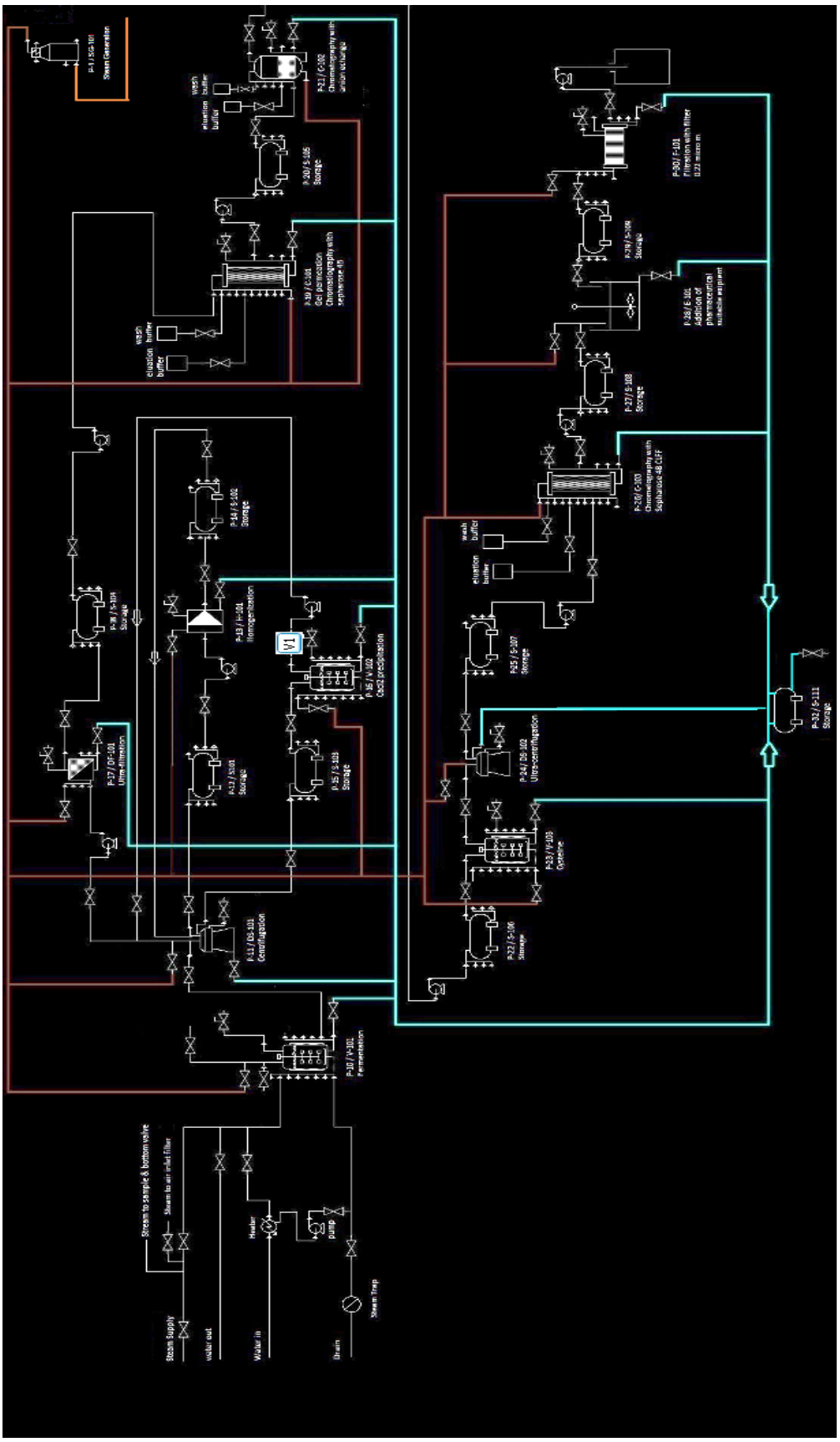


Figure 32 : *Button V1 Created*

Next step is creating label and collected it with buttons to show if we click the valve button will give "ON" or "OFF"

We need a code through which we can enter a condition and we can access to open-close the valve plus give in the label "ON"-"OFF", and turn off-on the pump plus give "ON"-"OFF".

This is the code:

```
          # Create Valves

             #V1
wx.StaticText(panel,-1," V1 ",(18,300))

self.button_V_1 = wx.Button(panel, -1, "V1", pos=(95,100),size=(20,20))
self.Bind(wx.EVT_BUTTON, self.V1, self.button_V_1)


def V1(self, event):
    valve_status_ON = 'ON'
    valve_status_OFF = 'OFF'

    if  self.Valve1.GetLabel()==valve_status_ON:
        self.Valve1.SetLabel(valve_status_OFF)
        wx.MessageBox("V1 is Open ","Open", wx.OK|wx.ICON_INFORMATION)
    else:
        self.Valve1.SetLabel(valve_status_ON)
        wx.MessageBox("V1 is Close","Closed", wx.OK|wx.ICON_INFORMATION)
```

Figure 33 : *Python 5*

With this code we have continued the Screen Controller to get it, this all buttons and labels represent the actuators, for the sensors we have developed some labels because the data of the sensors are taked from Velleman board.

We passed by some problems with function, how we will join the button with label and how to connect the label with condition 'if …else' to given "ON" or "OFF", and the order of steps. With the help from www.Python-forum.org, there we asked about the problem, and how to fix it.

They have given us help and repaired the code just in exception case.

This was the wrong code:

CODE: SELECT ALL

```
            #V1
      self.button_V_1 = wx.Button(panel, -1, "V1", pos=(95,100),size=(20,20))
      self.Bind(wx.EVT_BUTTON, self.V1, self.button_V_1)

def V1(self, event):

            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.V1.Bind("<Button-1>", callback):
            #self.V1 == True:
            #valve_status = valve_status_ON
             self.Valve1.SetLabel(valve_status_ON)
             wx.MessageBox("V1 is Open ","Open", wx.OK|wx.ICON_INFORMATION)
```

Figure 34: *Shot of the wrong code in forum.*

And this was the right code after the help from the forum python site:

CODE: SELECT ALL

```
def V1(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'

            if self.Valve1.GetLabel()==valve_status_ON:
                self.Valve1.SetLabel(valve_status_OFF)
                wx.MessageBox("V1 is Open ","Open", wx.OK|wx.ICON_INFORMATION)
            else: :
                self.Valve1.SetLabel(valve_status_ON)
                wx.MessageBox("V1 is Close","Closed", wx.OK|wx.ICON_INFORMATION)
```

Figure 35 : *Shot of the right code in forum.*

After writing the code for all the buttons, labels, text, we go the Controller Screen:

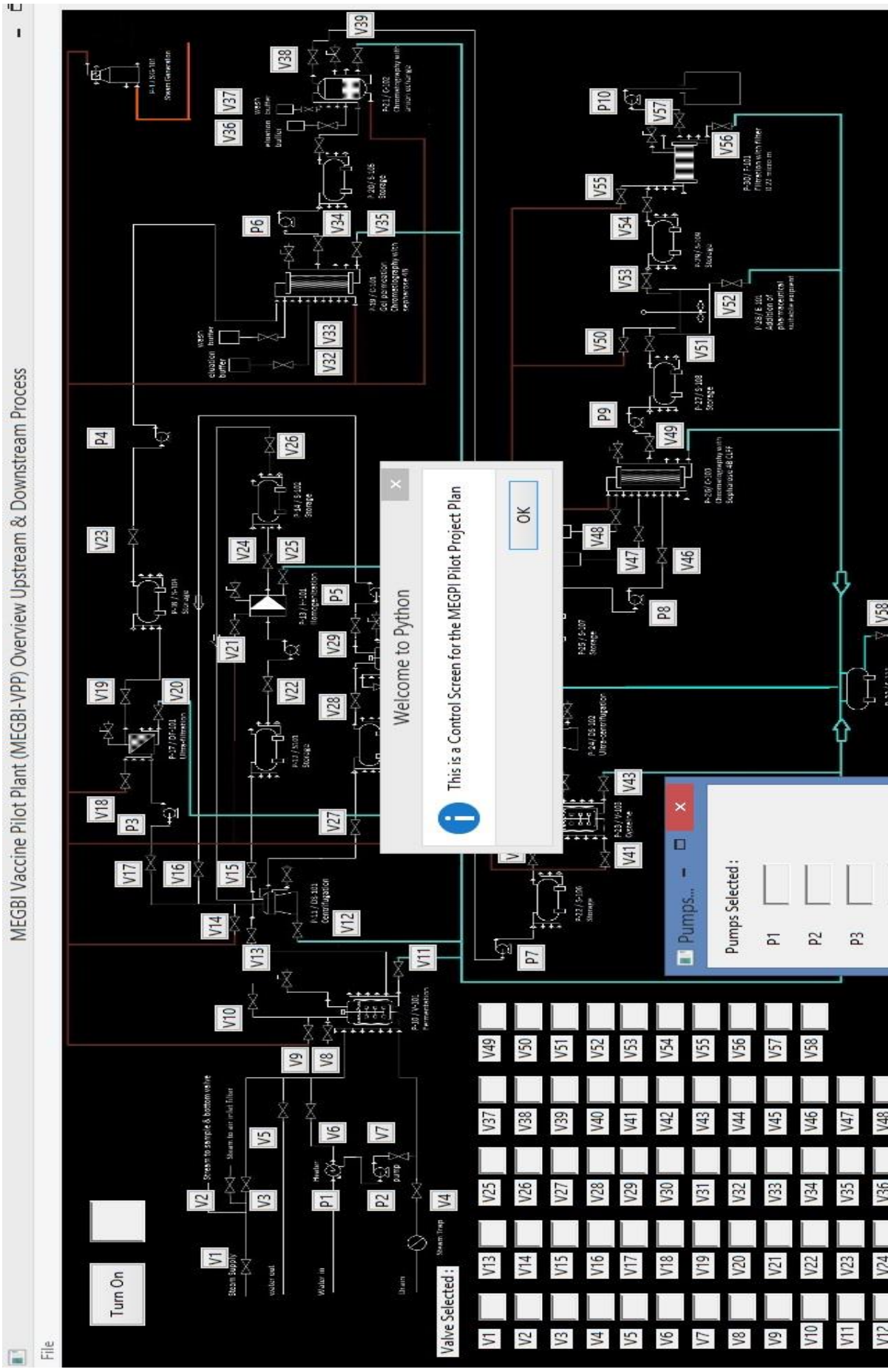


Figure 36 : *The control screen full of buttons ,labels ,list box.*

So as we talk before, the next step is connecting the control screen to USB Velleman K8061 board, we have used a specific code on Python to can the screen read the board and run it, before that we have defined the device of the USB board to pc by 2 dll files named "K8061.dll" " mpusbapi.dll" we downloading it from a site [7], when the board was connected to the screen we get a command shows the message between it (figure 2.3).

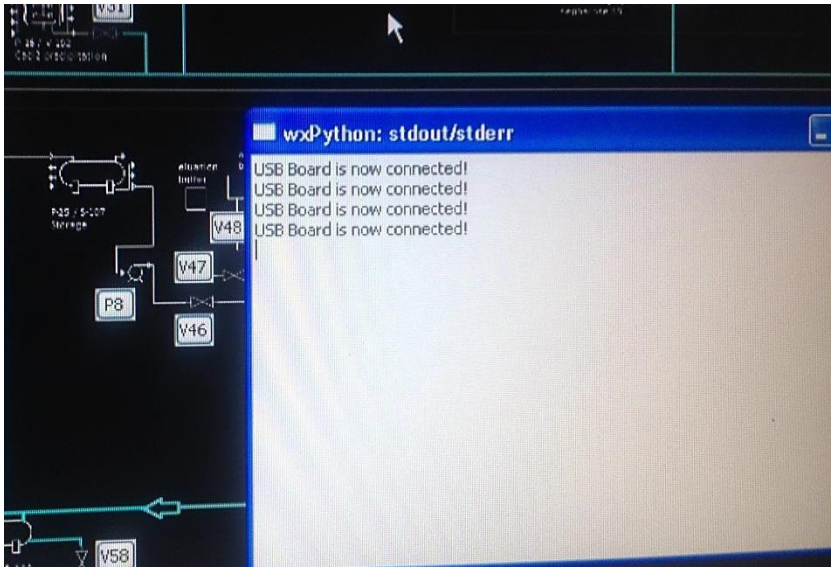


Figure 2.37 : *Command between the USB board and the control screen .*

This is the code:

```
#**********************                Run USB System                    **********************
#*****************************************************************************************************
    def OpenUSBBoardThread(self):
        self.dll = windll.K8061
        i = self.counterUSBBoards
        for doit in range(0,i+1):
            try:
                self.dll.OpenDevice()
                self.USBOpened = True
# debug info
                print ('USB Board is now connected!')
#end debug info
            except:
                txt = ('Please Check USB Board connection')
                print ('txt')
                return
```

Figure 38 : *Python 6*

Next of this code, must know how to connect the screen to the Velleman board, for that we must read about Velleman board, how to connect it ,how many inputs outputs ,the channels it has, and the actuators sensors which represents the outputs inputs .

And to connect the temperature sensors and get value, we must use a function to make the Velleman board read the value of the sensors and represents it in the label, to read the value should we use self.dll.ReadAnalogChannel(Adress,Channel) function ,the address of the board used is 0 and channel was 1. , next is the code:

```
        # temperature sensors value

wx.StaticText(panel,-1," Temperature Value ",(100,12))
self.temp_Vaporizer_out = wx.StaticText(panel, size = (26, -1), pos = (200, 10), style = LABELSTYLE )



new_value = str(self.dll.ReadAnalogChannel(0,1))
self.temp_Vaporizer_out.SetLabel(new_value)
self.temp_Vaporizer_out.Refresh()
```

Figure 39 : *Python 7*

After knowing how to run, we must collect the circuit of the temperature sensor:

16.3 Adaptation of Device Sensors and Actuators

16.3.1 Actuators (Valves)

Next we will talk about the actuators and sensors, and how we have connected it to the USB board to control them by the control screen. But when we makes the connection between the temperature sensors USB board we get wrong results that make a question about temperature so we have canceled it from the project and we continue about automation system by control the fluid flow from the actuators. To connect the actuators to USB board we need a relay, this relay will control the valve by open/close when they takes the order from the board same to pump by turn on/off, so we have built this relay by using a schema (figure 2.4).

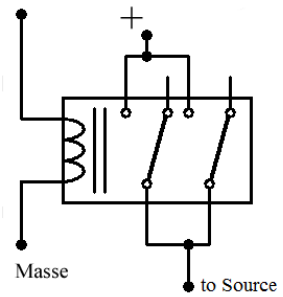
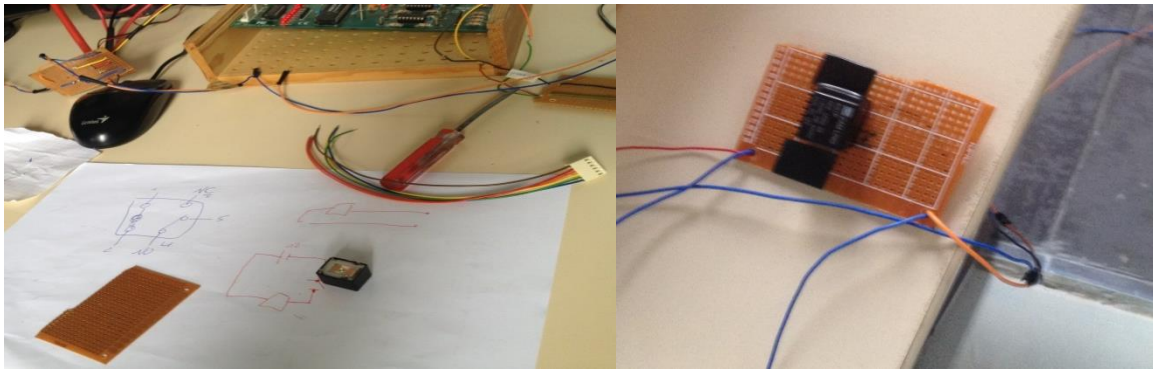


Figure 2.40:*Schema represents the construction of relay.*

So with this schema (figure 2.4), we build it by: relay-small board-tapes-welding



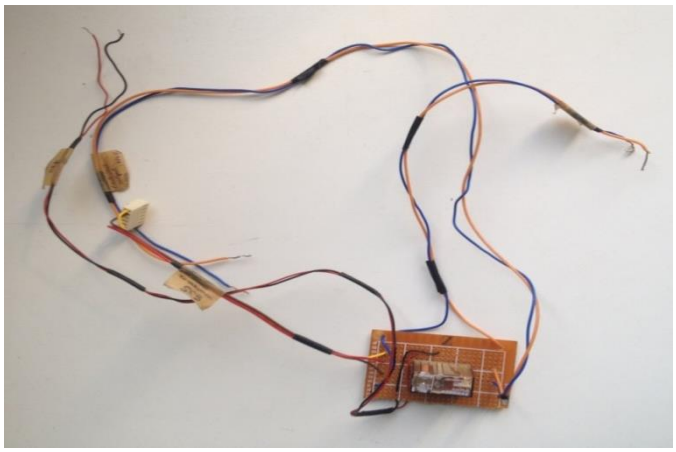
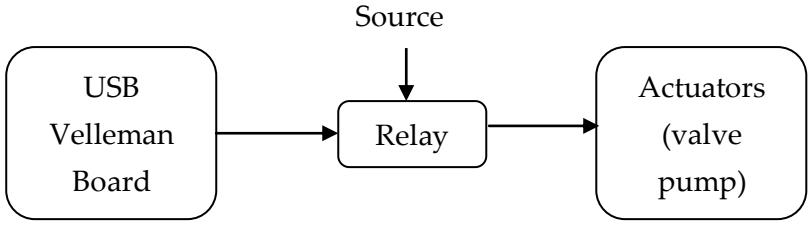


Figure 2.41: *installation a relay.*

And we use it to connect the actuators so as we can see on figure it's like a switch but with order this order come from the board, plus this actuators have connected to outputs (digital or analog).



4 steps to link the relay and make the connection between the board and the actuators:

- Connecting the relay to source (220V)

- Connecting the relay to actuators (220V)
- Connecting the relay to outputs channel(5V-12V)
- Connecting the relay to power of USB board(12V)

After knowing how to connect the actuators, we take a valve and we will try to connect it, so as we talk before, we have connected the valve to relay, then we have linked the relay and we get the circuit between the board, valve, relay, source (figure 2.6).

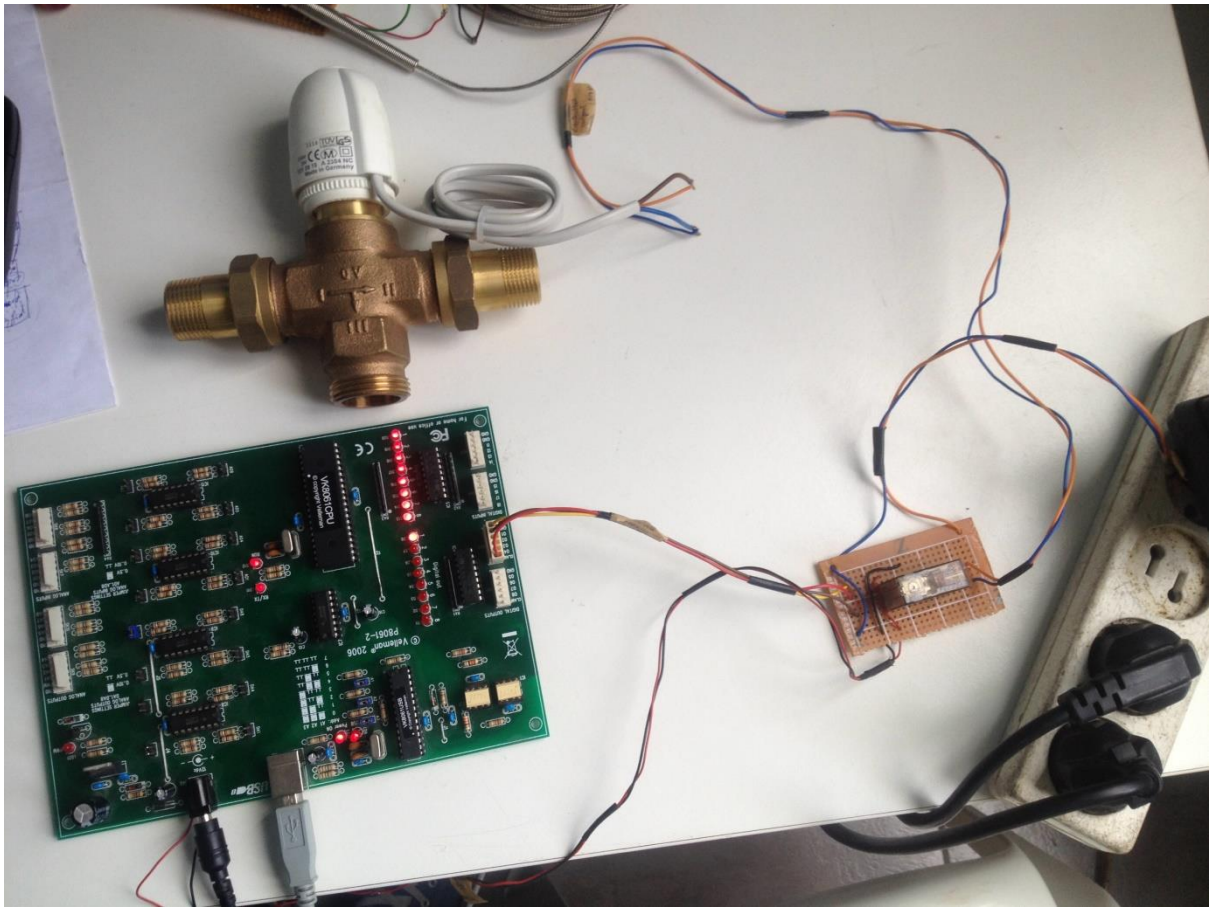


Figure 2.42 : *The circuit between the board, valve, relay, source.*

And we will test it to make sure the valve the circuit is working good and ready to use it in the test stand, so we will try to open and close it from the screen by clicking on button and we take many pictures showings the test, as we see the blue color mean the valve is getting open so the order from the board has been arrived to valve to open and the red light on the board in the outputs channel confirm the same thing (figure 2.7) ,and vice versa the red light disappeared when we close the valve so that confirm the same thing that the board gives the order to open or close(figure 2.8), so the control of the valve is working .

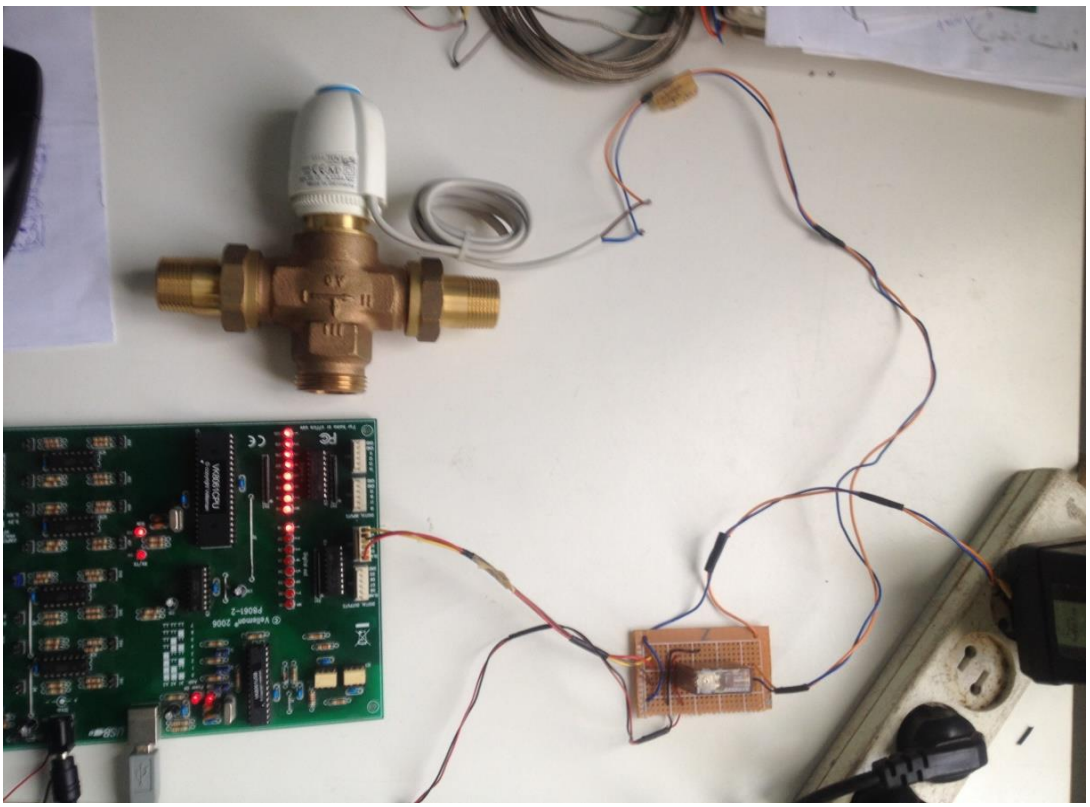


Figure 2.43 : *The circuit when the valve gets opened.*

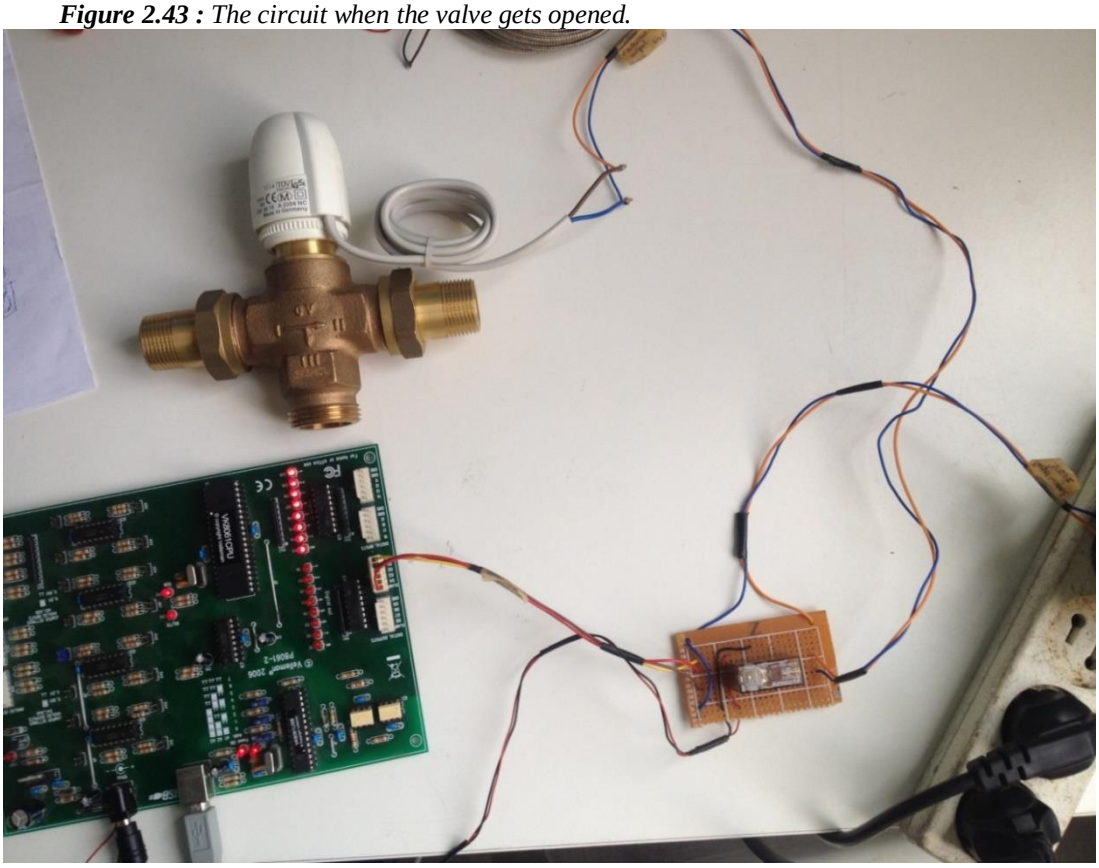


Figure 2.44 : *The circuit when the valve get closed.*

So we have 58 valves and 10 pumps concerning the production,and the valves , the pumps have used on temperature control system for bioreactor and the cleaning way,and we will make a connection on the PI diagram between the all actuators and the boards ,PLC.so we have connected each valve to a output channel on board or PLC(figure 2.9),16 outputs (digital + analoge) on board ,16 on PLC.

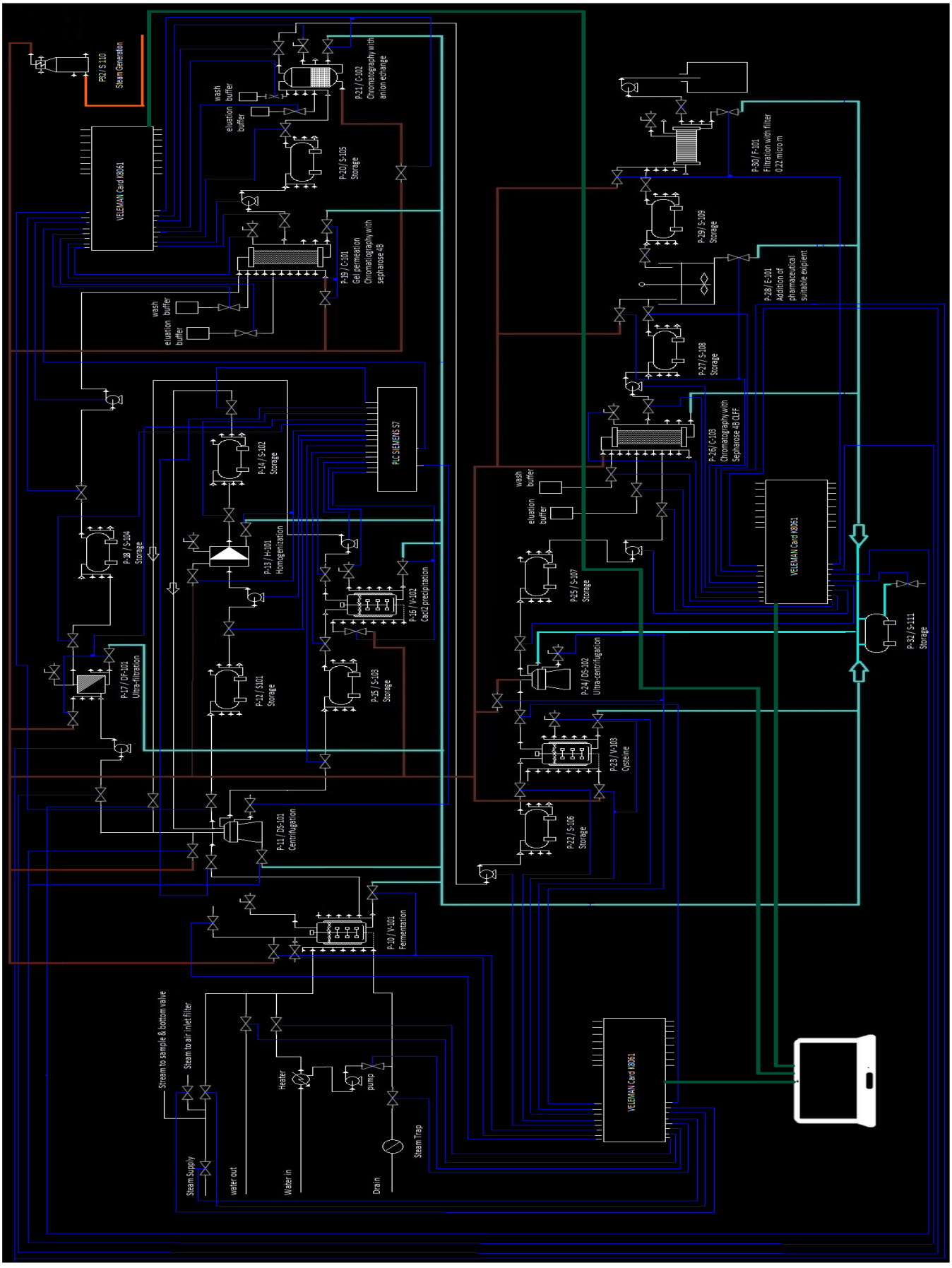

P32 / S-110
Steam Generation
VELEMAN Card K8061
wash buffer
elution buffer
P-21 / C-102
Chromatography with anion exchange
P-20 / S-105
Storage
P-19 / C-101
Gel permeation Chromatography with sepharose 4B
P-30 / F-101
Filtration with filter 0.22 micro m
P-29 / S-109
Storage
P-28 / E-101
Addition of pharmazeutical suitable exipient
P-27 / S-108
Storage
P-26 / C-103
Chromatography with Sepharose 4B CLFF
PLC SIEMENS S7
P-14 / S-102
Storage
P-38 / S-104
Storage
P-13 / H-101
Homogenization
P-16 / V-102
Cacl2 precipitation
P-17 / DF-101
Ultra-filtration
P-12 / S101
Storage
P-15 / S-103
Storage
P-11 / DS-101
Centrifugation
P-25 / S-107
Storage
P-24 / DS-102
Ultra-centrifugation
P-23 / V-103
Cysteine
P-22 / S-106
Storage
P-31 / S-111
Storage
P-10 / V-101
Fermentation
Stream to sample & bottom valve
Steam to air inlet filter
Heater
pump
Steam Supply
water out
Water in
Drain
Steam Trap

Figure 2.45 : Network between the actuators and the boards,PLC.

16.3.2 Sensors

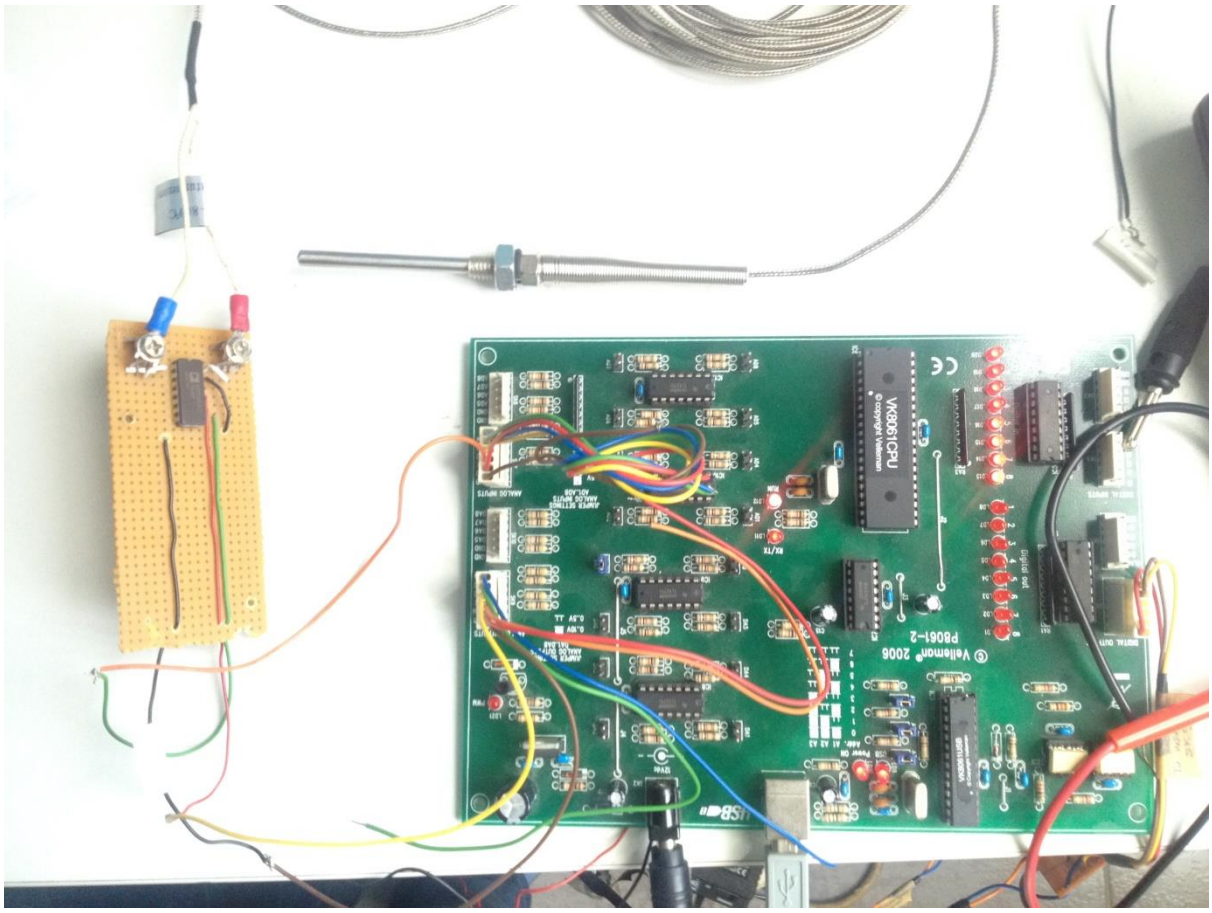


Figure 46 : *The circuit of Temperature sensor with Velleman board.*

This is the circuit, in which we have connected the temperature sensor to Velleman board across a connection, we have put it on inputs channel one but we have to connect it to output at same time, for even take electricity from that output it just the only way to filled.

So we need 2 connections, one on inputs channel 1 position 1, and other in outputs channel 2 position 1 plus ground and it's ready to work.

After the scan and test shows that there is the temperature sensor malfunction and it should be removed from the project for a reason of time constraints.

We take 3 shots to show how the sensor didn't give a constant value this shows that there malfunction, plus all the connection was right.

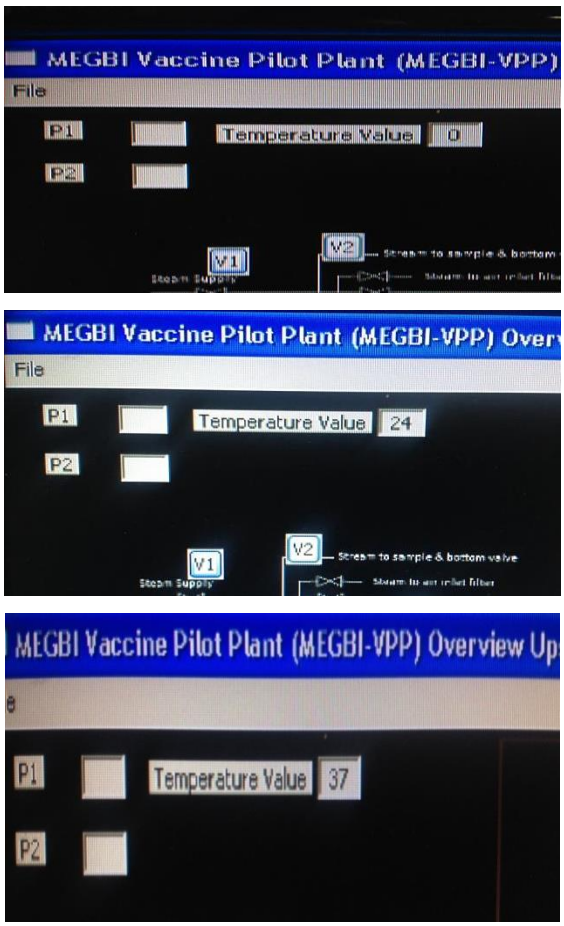


Figure 47 : *3 Shots show the malfunction.*

Now, how to connect the outputs represents across valves and pumps.

For example we worked on valve, must click on V1, that button give order to Velleman board to open or close the valve. So we have used a code to connect the python code valve to the board.

This is the code:

```
    def V1(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'

# open the USB board

        if  self.Valve1.GetLabel()==valve_status_OFF:
            self.Valve1.SetLabel(valve_status_ON)


            self.OpenUSBBoardThread()
            self.dll.SetDigitalChannel(0,1)
            wx.MessageBox("V1 is open ", "Open", wx.OK|wx.ICON_INFORMATION)
            self.timer = wx.Timer()
            self.timer.Bind(wx.EVT_TIMER, self.on_timer)
            self.timer.Start(0)
            wx.MessageBox("V1 is Open ","Open", wx.OK|wx.ICON_INFORMATION)
        else:
            self.Valve1.SetLabel(valve_status_OFF)
            time.sleep(2)
            self.dll.ClearDigitalChannel(0,1)
            print 'Digital Channel Cleared, V1 turn off'
            wx.MessageBox("V1 is Close","Closed", wx.OK|wx.ICON_INFORMATION)
```

Figure 48 : Python 8

We have used timer to get order fast if we click it opens directly and same to close it.

Now if I click on valve V1 they give V1 is open and the board take the order to open it, same to close it.

How to connect the valve to the Velleman board:

There should be a relay, like we see:

Must make a relay, we start with collect the contents

Needed:

- relay
- Small board
- Tapes
- Welding

the relay play the role of the brood , but with order.

For example , if a request from the board to open the actuators ,this brood will hatched and give the power required to actuators.

So when we click on button at screen,the board get order to open or close the actuators,and the relay is helping to do this order,across a signal from the board represent by 0.5V.

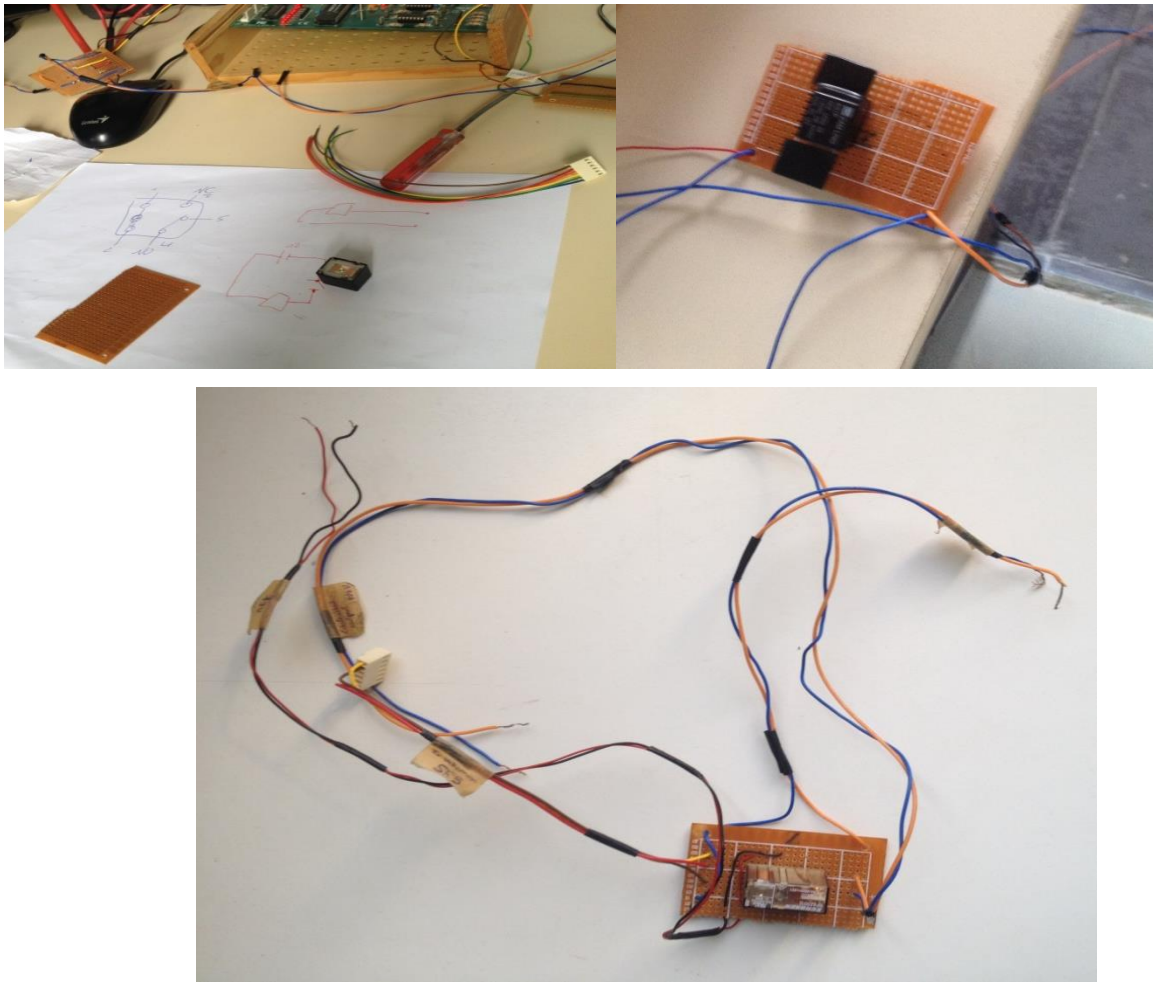


Figure 49: *Building a relay.*

This work like an electric transformer:

4 tapes:

- First one is getting 220V from source.
- the other is 220V linked to Valve(because the valve working on 220V)
- the other is connected to the source of Velleman board that mean take 12V
- The last one is connected to channel Analog outputs.

And we take a picture of the circuit:

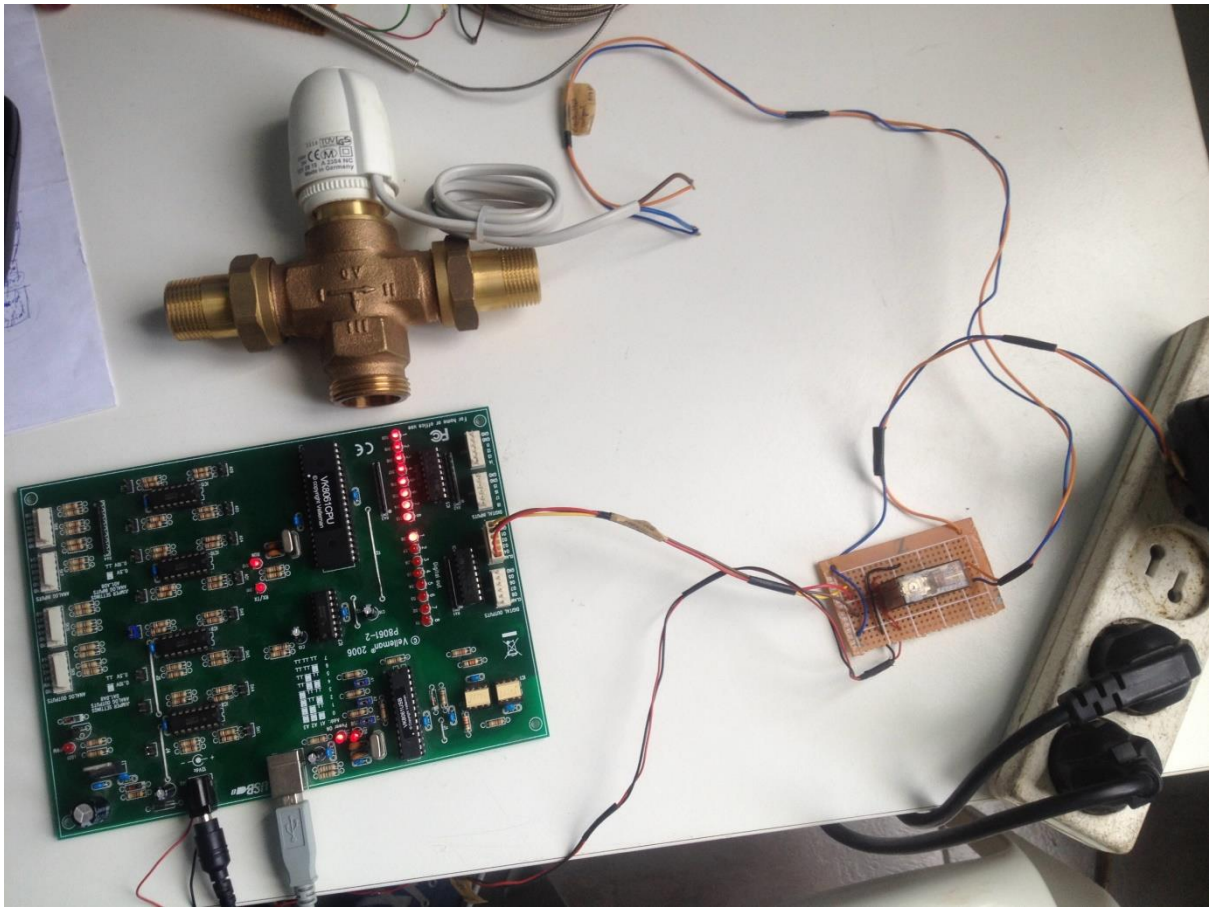


Figure 50 : *The circuit of Valve with Velleman board*

Now must test it:

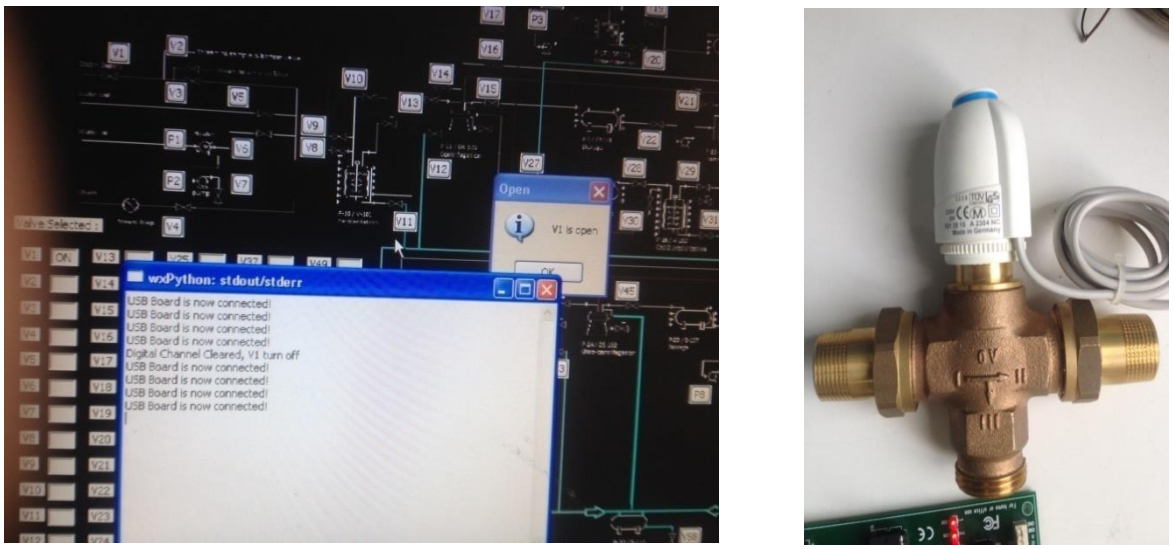


Figure 51 : *Testing the process.*

The valve is open, that blue color on valve means that the valve was open.

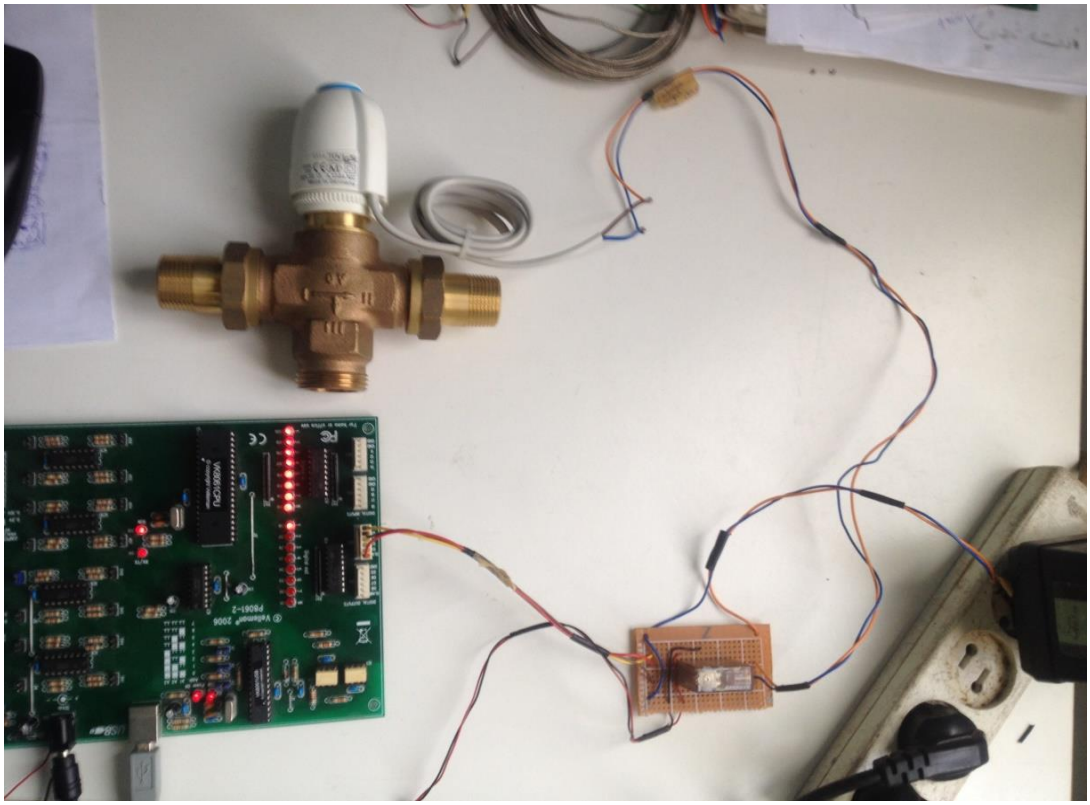


Figure 52 : *The circuit when the valve gets opened.*

They is red light on near the series of light, that light meaning the valve is getting order and open that is working when The button was clicked, so the valve was open.

Next click to close it, and we should showing it too:

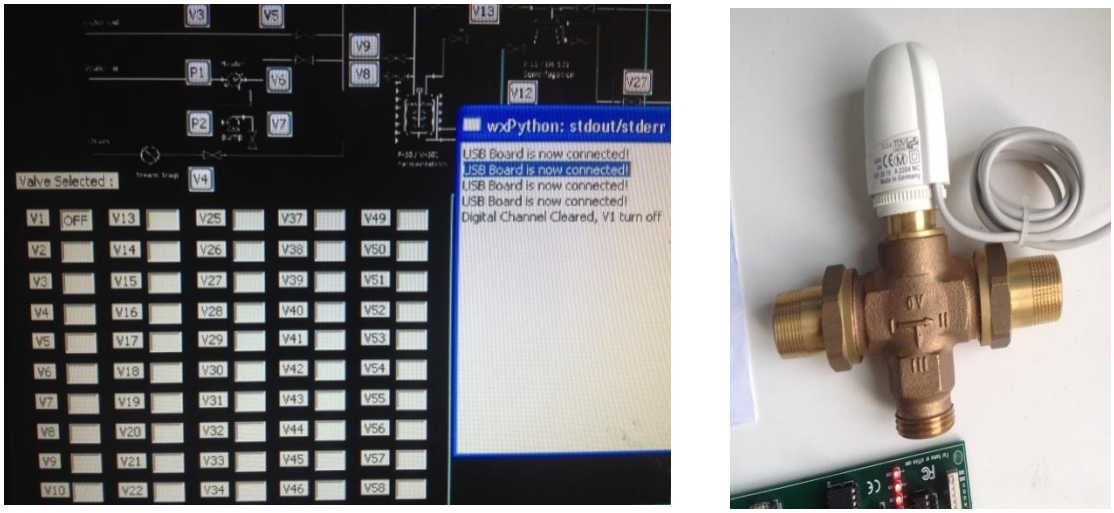


Figure 53 : *Testing Process.*

The valve is closed; we didn't saw the blue color.

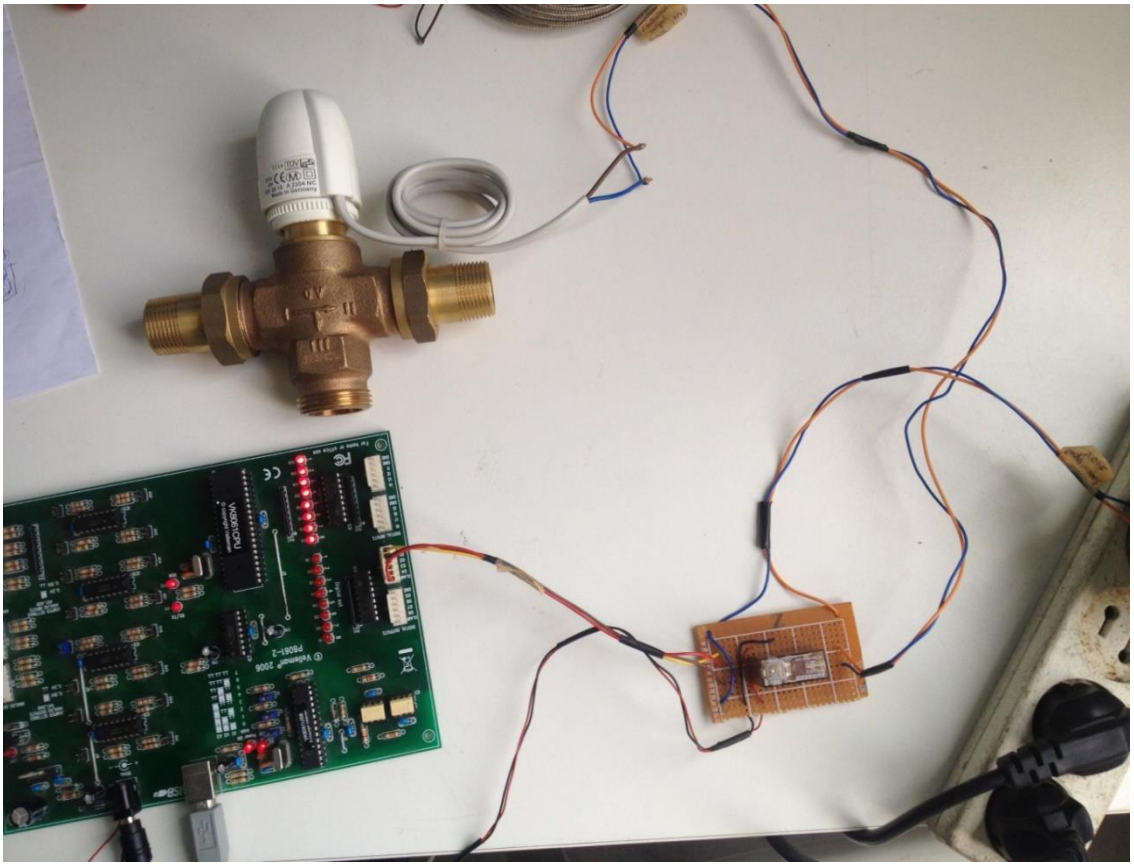


Figure 54 : *The circuit when the valve get closed.*

We saw in this picture the red light is off, that means the board gives order to valve to turn off, and is sawing in screen V1 "OFF".

Next, we have moved to the test stand, we have painted the circuit needed, across a website [2].

We have taken part of The Downstream processing, and we will test it across the board, valve, pump, and the control screen.

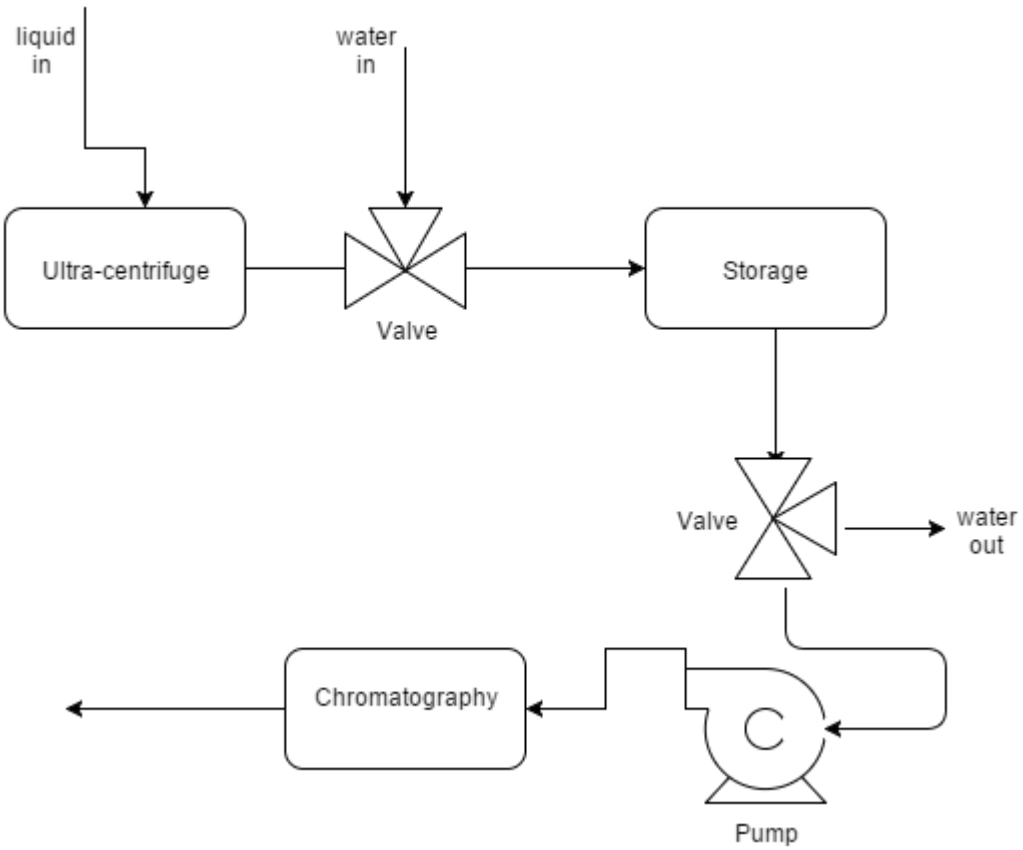


Figure 55 : *Diagram for the part of downstream processing used.*

We saw liquid enter to the ultra-centrifuge and continue across the valve to Storage.

After the storage, the liquid continue to Chromatography across valve and pump.

We saw in the way a line to water-in, that line is used to clean the storage, and more the line of water-out is used to get rid of the dirty water.

Across the diagram we will create a schema to the process that will test it.

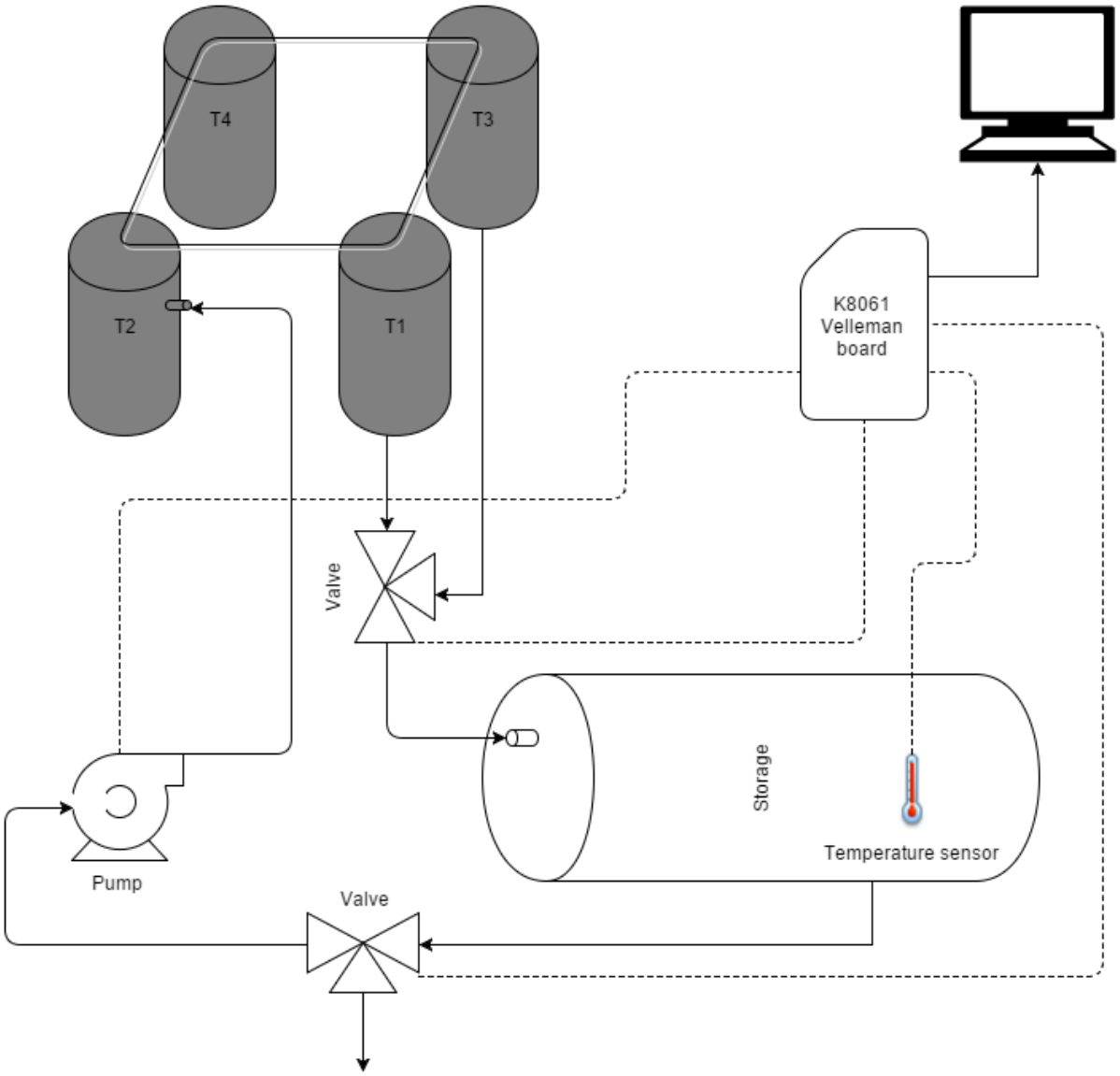


Figure 56 : *Schema of the part of downstream processing used.*

The contents of this schema:

- T1 represent the ultra-centrifuge.
- T2 represent the Chromatography.
- T3 represent the Storage of water used for cleaning.
- USB Velleman K8061 board connected to laptop.

This schema is a miniature of the really circuit.

Figure 57 : *Test stand.*

Like we saw in schema we have built the test stand:

The board is connected to pc, and the control screen was working and ready to test,

With using of plastic pipes, we have linked T1 to valve and the valve to the storage, plus T3 to the valve too.

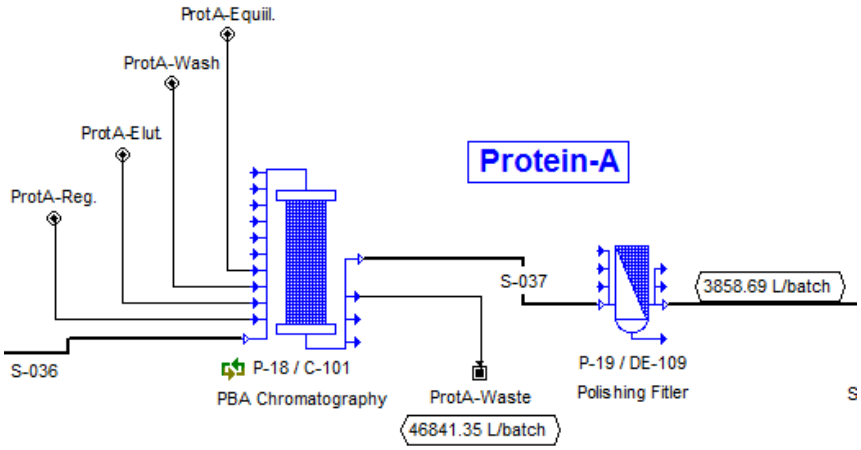
On the other we have linked the storage to valve, the valve to pump and pump to T2.

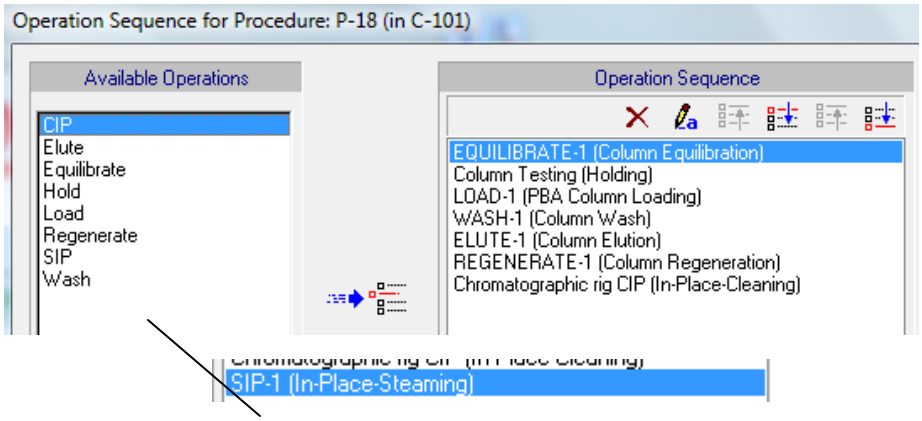
We have worked hard to build it, like we saw, we used plastic pipes, the mechanics tools, cash cables …

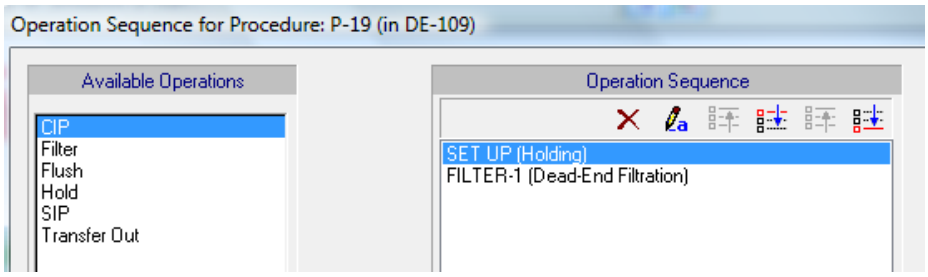
Finally getting the test stand:

Figure 58 : *The test stand ready for work.*

16.4 Functional Operation Algorithm of Automation System







Now we have created a algorithm represents the production of the vaccine process by process plus the valve necessary and the order of open/close turn on/off for the process and we have based on this algorithm to made the full automation of the system of production the vaccine by control the fluid flow, so we have based on this algorithm to collect on Python[8] a code can with it "Turn On' the system to start the production, so with the algorithm we have created the Python code that we have need to the automation process and it's done ,ready to test , just click on "Turn On".

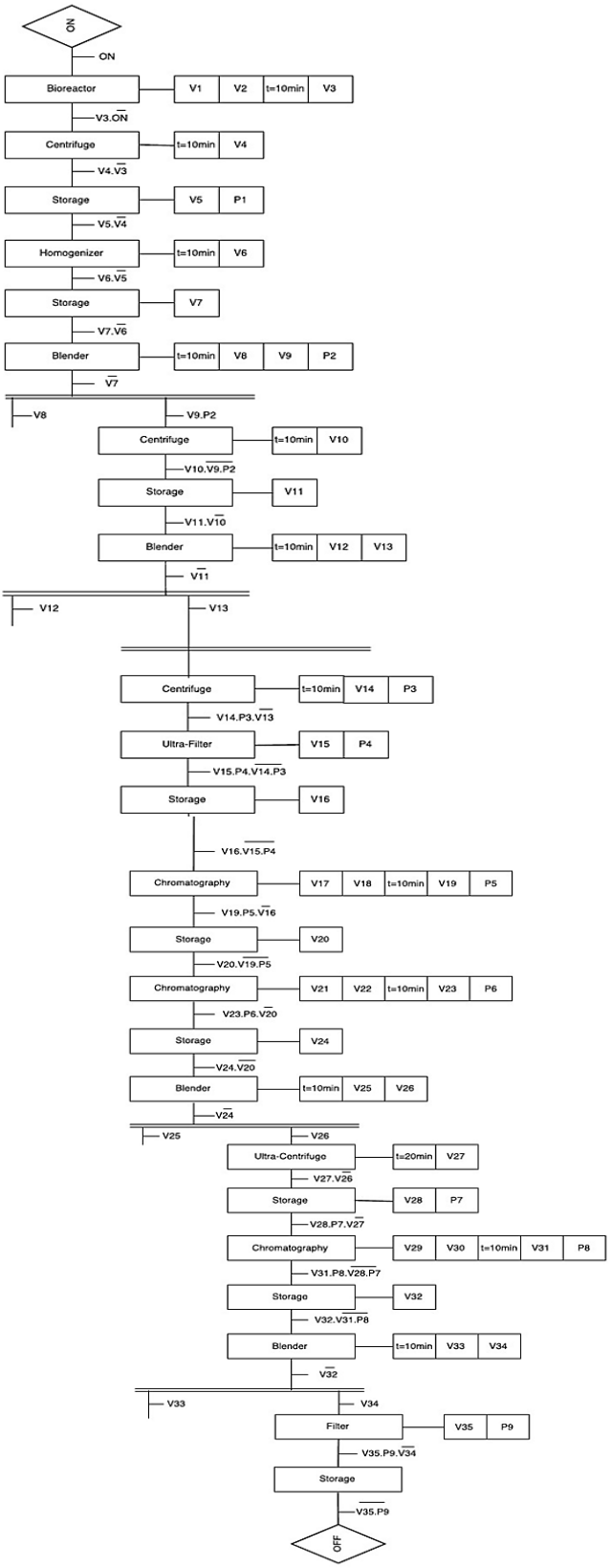
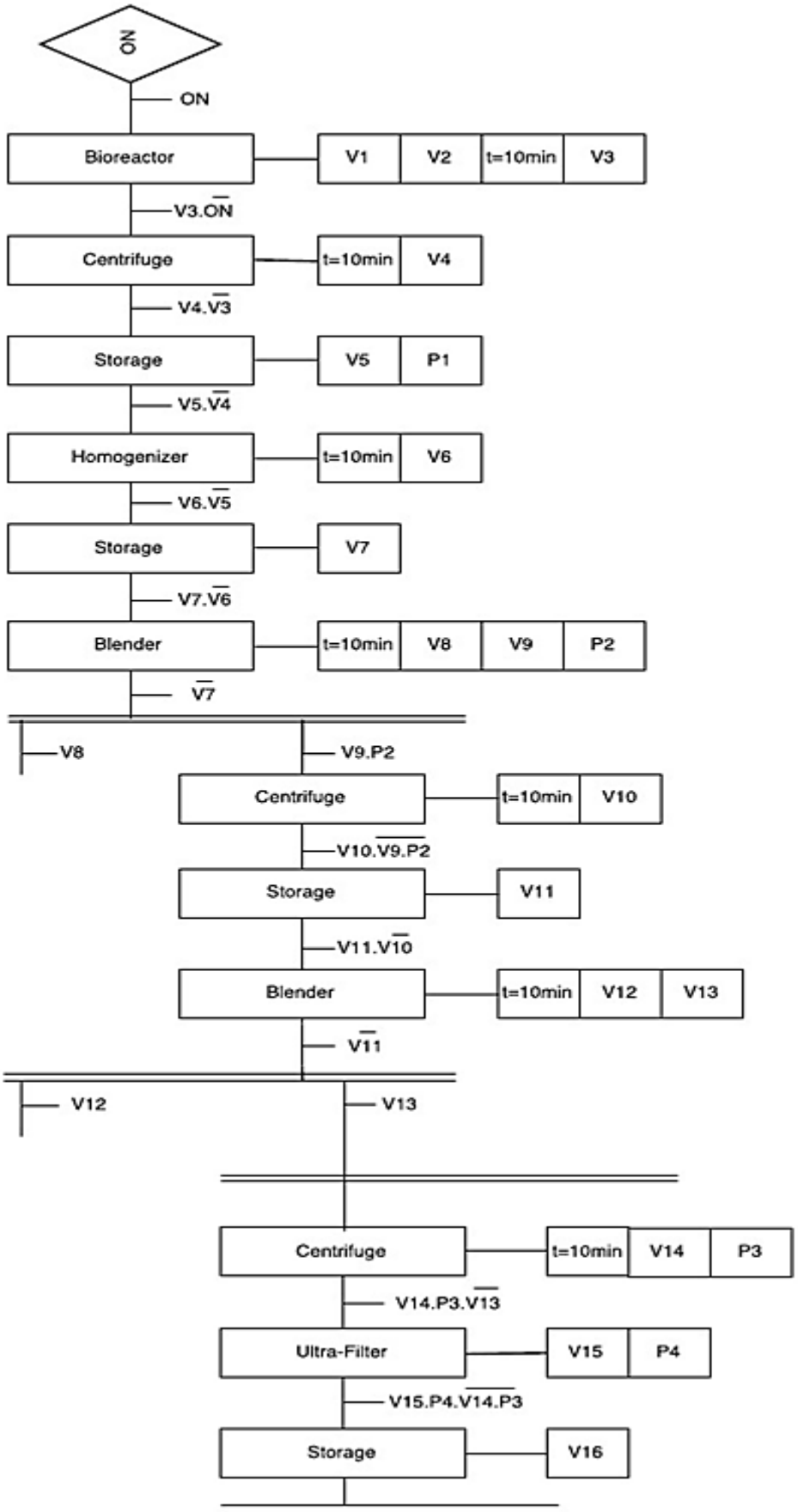


Figure 2.59a : *Algorithm represents the automation of the production (Overview)*


ON
ON
Bioreactor
V1
V2
t=10min
V3
V3.$\overline{ON}$
Centrifuge
t=10min
V4
V4.$\overline{V3}$
Storage
V5
P1
V5.$\overline{V4}$
Homogenizer
t=10min
V6
V6.$\overline{V5}$
Storage
V7
V7.$\overline{V6}$
Blender
t=10min
V8
V9
P2
$\overline{V7}$
V8
V9.P2
Centrifuge
t=10min
V10
V10.$\overline{V9.P2}$
Storage
V11
V11.$\overline{V10}$
Blender
t=10min
V12
V13
$\overline{V11}$
V12
V13
Centrifuge
t=10min
V14
P3
V14.P3.$\overline{V13}$
Ultra-Filter
V15
P4
V15.P4.$\overline{V14.P3}$
Storage
V16

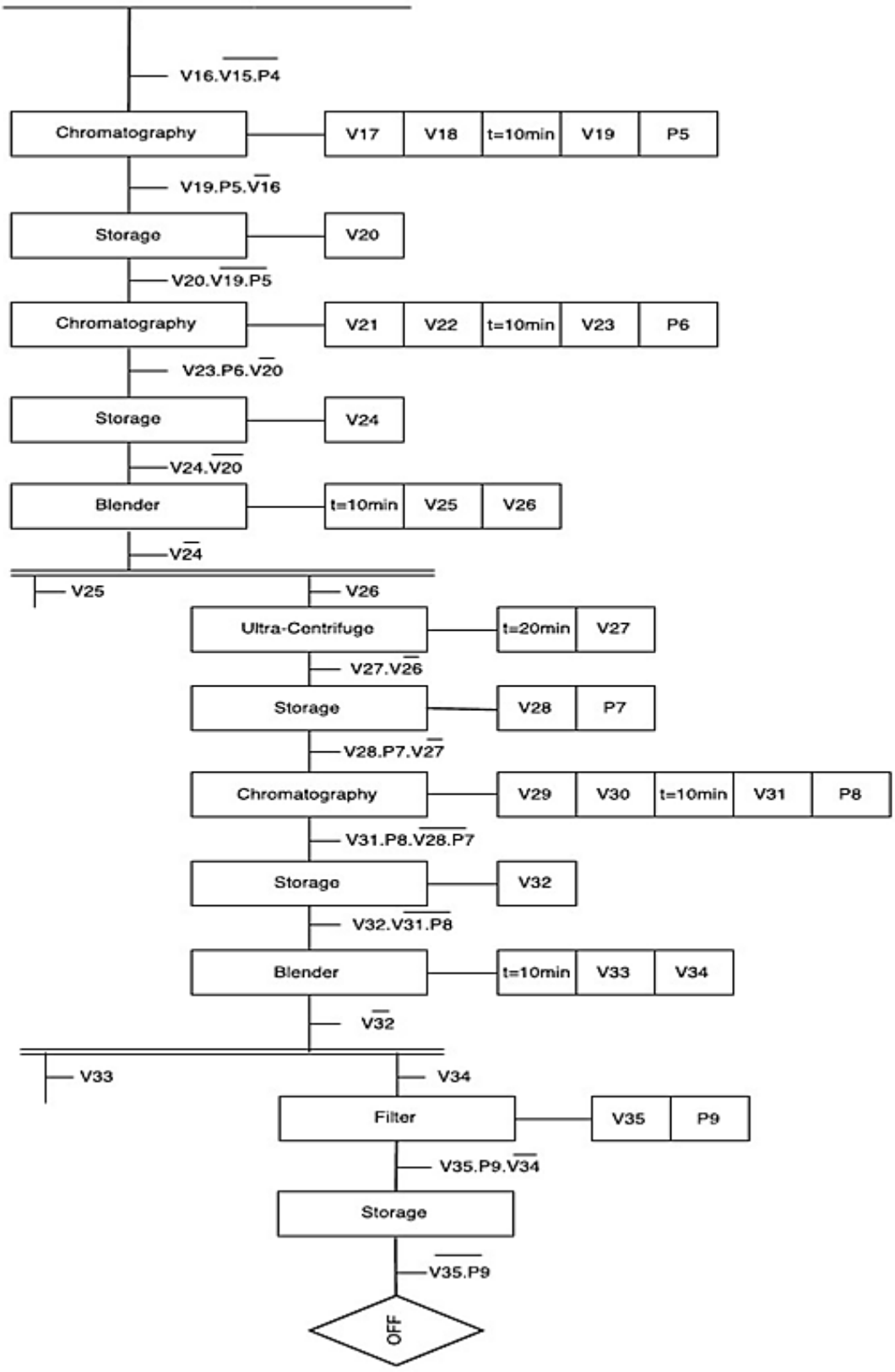


Figure 2.60b : *Algorithm represents the automation of the production (as in overview, but two pictures)*

After creating that algorithm, we have moved to the test stand, the idea of this step is to build a part of the PI diagram of the MEGBI-VPP Processing and testing it ,so we have to

choose the circuit that we will based on it build the test stand. By a website [9] we have created the diagram that represents this part. We will test it by the board, valve, pump, and the control screen.

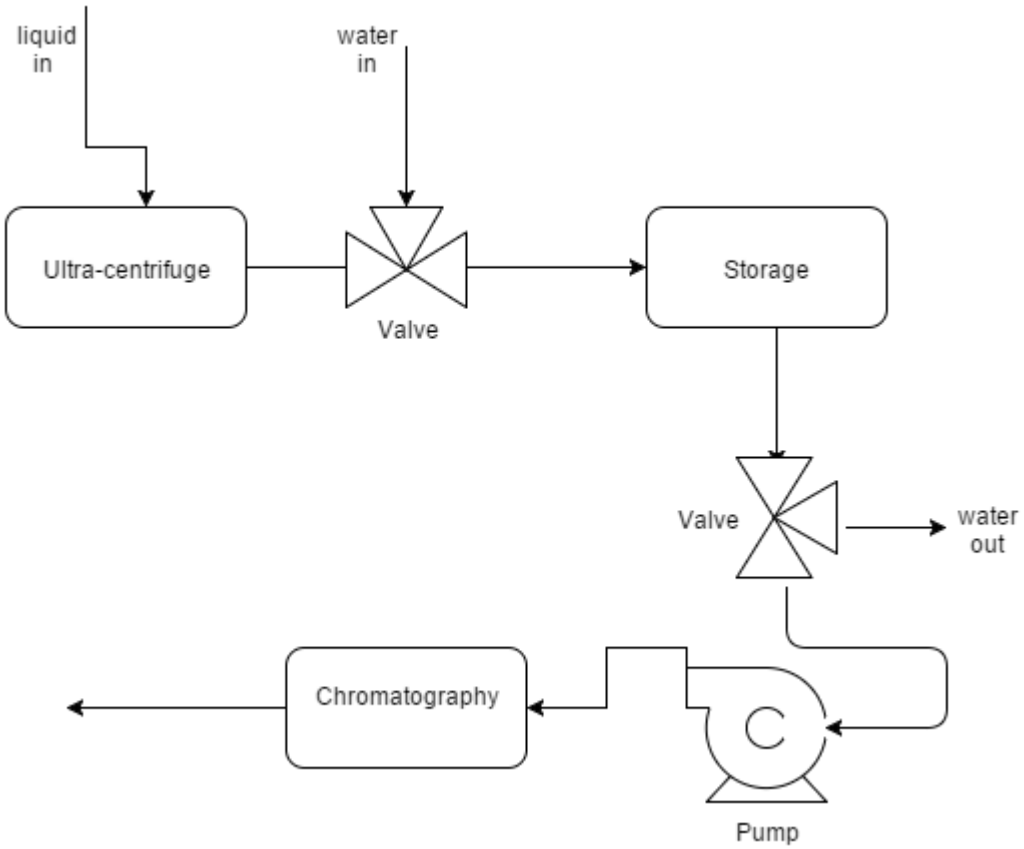


Figure 2.61 : *Diagram for the part of MEGBI-VPP Processing used.*

The liquid enter to ultra-centrifuge and continue by a valve to Storage, then to Chromatography by valve and pump (figure 2.11). This liquid represents the production and this is the part that we have chosen to base on it to build our test stand. We saw in the way a line to water-in that line is to clean the storage, and more the line of water-out is to get rid of the dirty water. With this Diagram we have created a Schema represents the test stand that we will build and each machine, instruments have to use (figure 2.12).

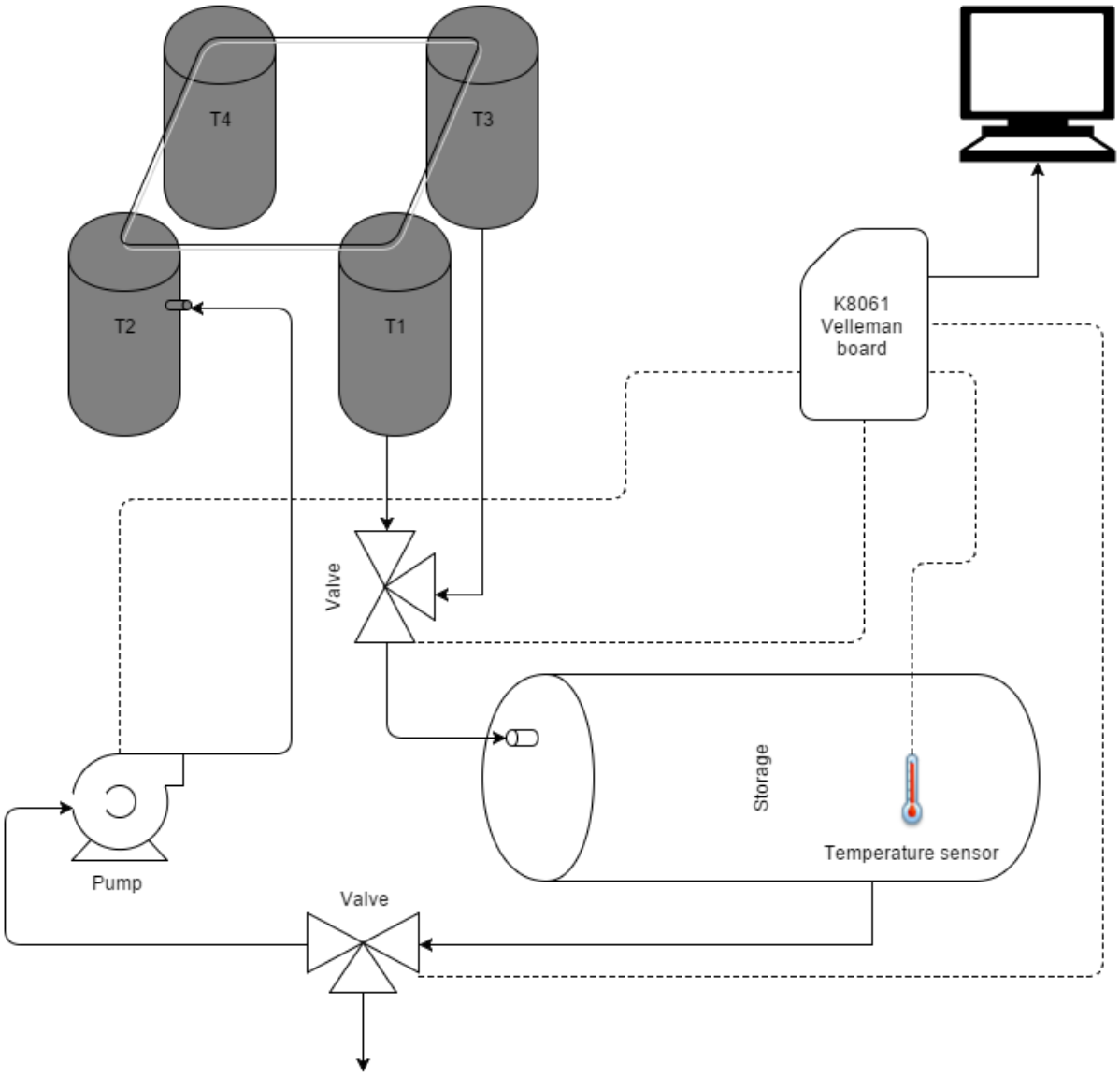


Figure 2.62 : *Schema for the part of the MEGBI-VPP Processing used.*

The contents of this schema:

- T1 represent the ultra-centrifuge.
- T2 represent the Chromatography.
- T3 represent the Storage of water used for cleaning.
- T4 is not used.
- USB Velleman K8061 board connected to laptop.

This schema is a miniature of the really circuit that we will build a really one like it.

Figure 2.63 : *Test stand.*

As we saw in schema we have built the test stand (figure 2.13). The board is connected to pc, and the control screen was working and ready to test, with using of plastic pipes, we have linked T1 to valve and the valve to the storage, plus T3 to valve too, T4 is not used. On the other we have linked the storage to valve, the valve to pump and pump to T2, here we have used a pump cause we want to send the production to T3 and T3 is located at a height of 2 meters We have worked hard to build it, like we saw we use plastic pipes, the mechanics tools, instruments, cash cable …

Figure 16.14 a: *The test stand after build it.*

Figure 16.14b: Installed *Electrical Control Cabinet*

17 Testing the automation system

We have divided our work to tow part of results, the first one will examine all the actuators in the test stand to see if there is any problem or to see if everything is all right, it has been working on three tests by trying to control the actuators on the test stand from the control screen, then the other will examine the full automation system of the production of the vaccine by testing the Python code that based on algorithm to do this automation and control all the process of the production.

17.1 Testing the Test Stand

We start with the test of the test stand we made three tests:

17.1.1 Test 1

On this test ,will examine the valve by sending water from the ultra-centrifuge to the storage and will exercise control over them and see if we can introduce water into the storage when we open the valve by pressing the button of valve on control screen and the usb board will give the order to open.In short,will click the button of valve on screen to open it and other click to close it.

Figure 3.1 : *The Test 1 concerning open and close a valve.*

PRECONDITION	TEST ACTIVITY	POST CONDITION	TEST SUCCEED/FAILED
PIPES WITHOUT WATER	OPENING AND CLOSING THE VALVE.	FIRST CLICK VALVE OPEN	SUCCEED

		OTHER CLICK VALVE CLOSE	

17.1.2 Test 2:

On this test ,will examine the valve with the pump by sending water from Storage to the Chromatography and will exercise control over them and see if we can introduce water into the Chromatography when we open the valve and turn on the pump,used pump here because the slot was from under so the liquid haved a low pressure,plus the Chromatography exist at high so must use pump to increase pressure.By pressing the button of valve with pump on the control screen and the usb board will give the order to open and turn on the pump.In short,will click the button of valve with pump on screen to open it ,turn on the pump and other click to close it and turn off the pump.

Figure 3.2: *The test 2 concerning open/close valve turn on/off pump.*

PRECONDITION	TEST ACTIVITY	POST CONDITION	TEST SUCCEED/FAILED

PIPES WITHOUT WATER	OPENING AND CLOSING THE VALVE WITH TURNING ON AND OFF THE PUMP.	FIRST CLICK VALVE OPEN AND PUMP TURN ON OTHER CLICK VALVE CLOSE AND PUMP TURN OFF	SUCCEED

17.1.3 Test 3:

In this test ,will examine the two previous two tests together.

Figure 3.3: *The Test 3 concerning open/close 2 valves and turn on/off pump.*

PRECONDITION	TEST ACTIVITY	POST CONDITION	TEST SUCCEED/FAILED
PIPES WITHOUT WATER	OPENING AND CLOSING THE VALVE(V1) WITH TURNING ON AND OFF THE PUMP.	VALVE1 OPEN PUMP TURN ON VALVE 2 OPEN	SUCCEED
	OPENING AND	VALVE 1 CLOSE PUMP TURN OFF	SUCCEED

	CLOSING THE VALVE (V2) AT SAME TIME.	VALVE 2 CLOSE	

17.2 Testing the full automation of the MEGBI-VPP Processing:

On this part we will test the automation system that we have created before and get the results about it, this automation is for the production only so they haves the valves and pumps necessary for it , we not including the valves necessary to cleaning.so as we see on algorithm before we have 35 valves 9 pumps so we needed 42 outputs ,divided on 3 boards each one have 16 outputs(digital analogue),but we will test the automation on board and screen cause we haven't the 35 valves 9 pumps so test the automation system by the board and the screen is enough cause that mean we will test the outputs and it's enough. Now we will shows all the results on list of pictures started by turning on the system by click on "Turn on":

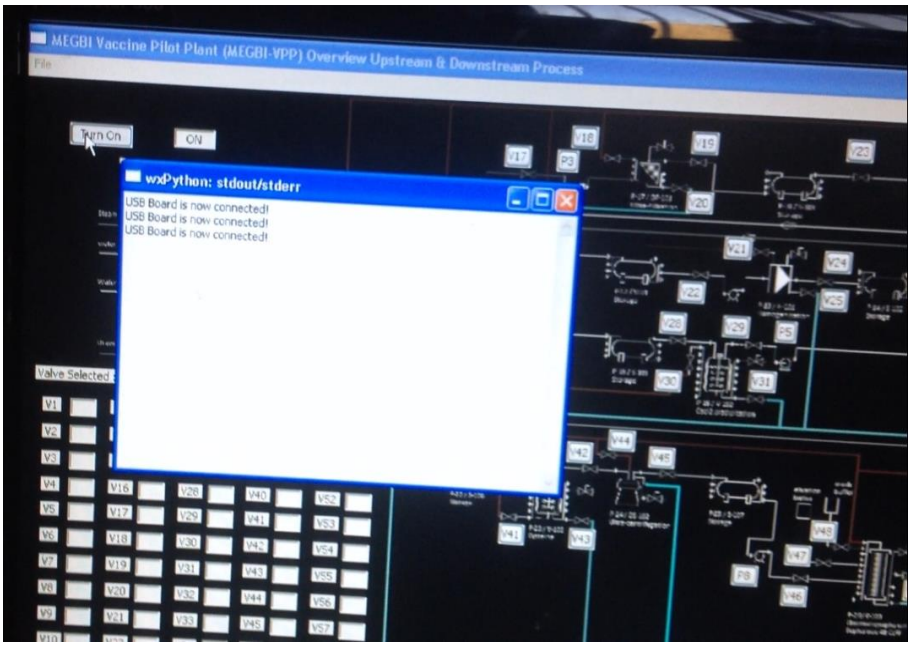


Figure 3.4: *The test of the automation system by Turn on.*

With clicking on "Turn On", the system is start (figure 3.4), and the full automation is started to test, this full automation can gives to us the controls of the fluid flow.

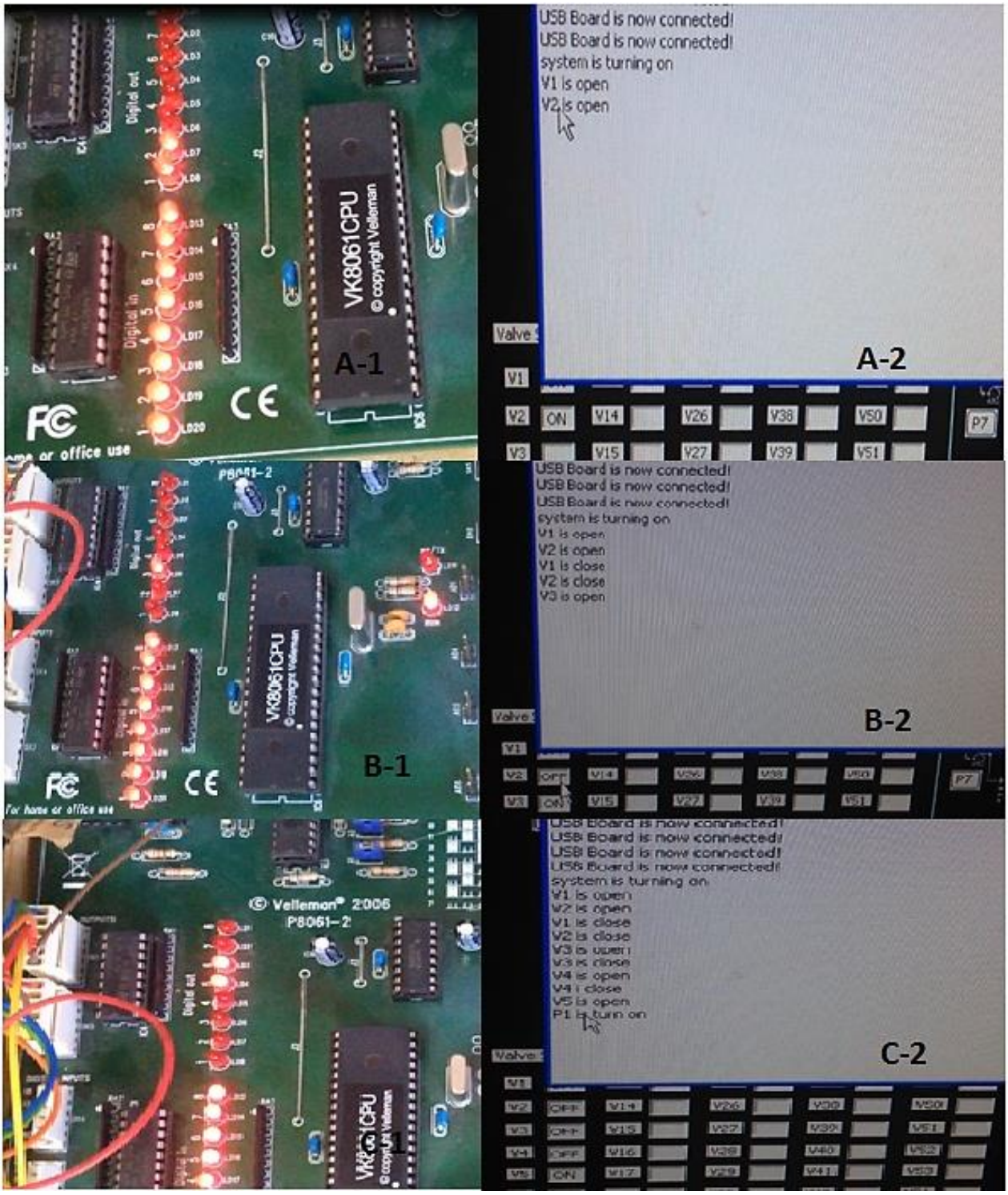


Figure 3.5: *The test of the full automation system step A,B and C.*

The automation is working good, as we see (figure 3.5) the valve V1 is open and the red light on board mean that , so when it close this red light will disappeared and that what we shows in this picture

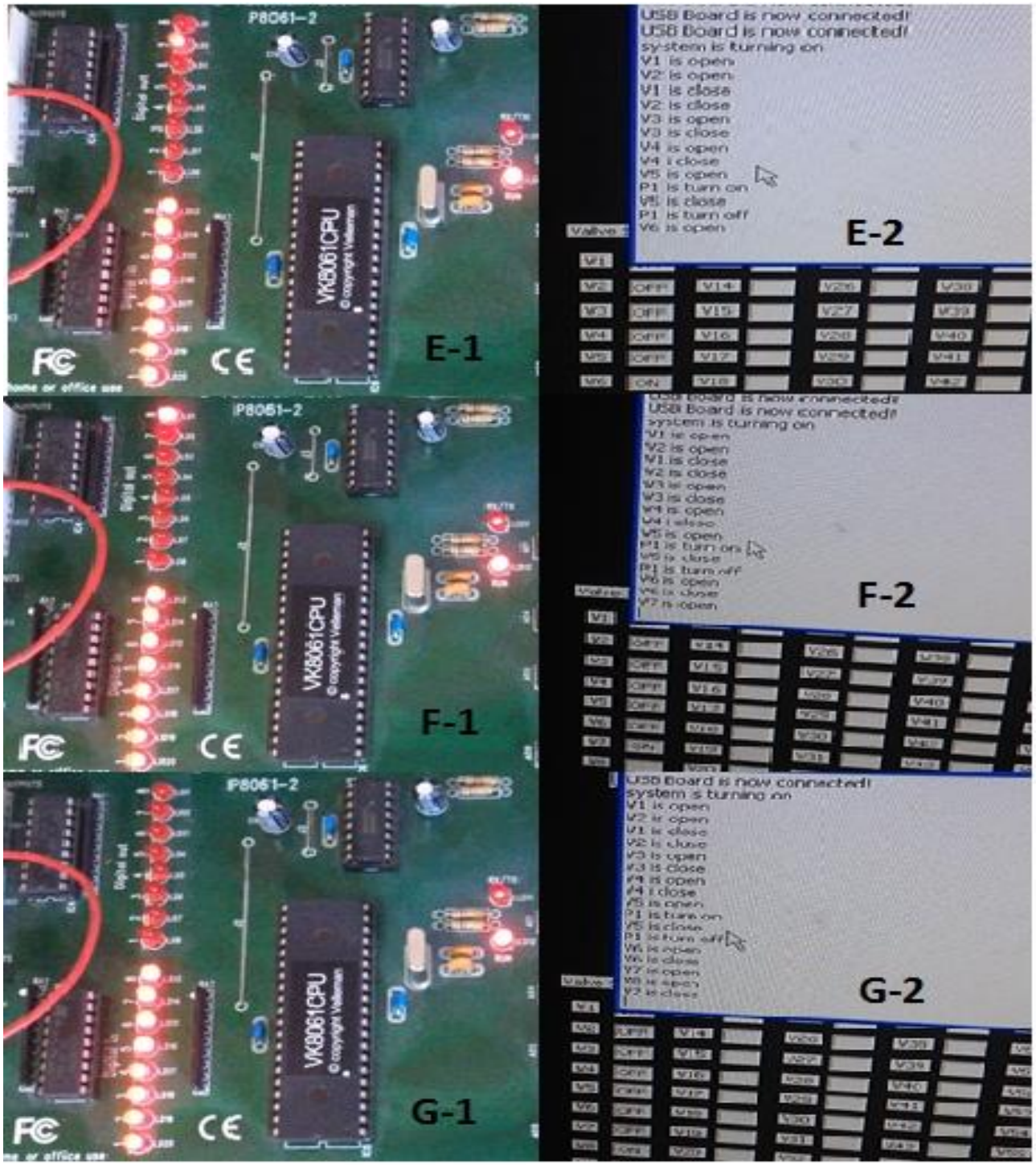


Figure 3.6: *The test of the full automation system step E,F and G.*

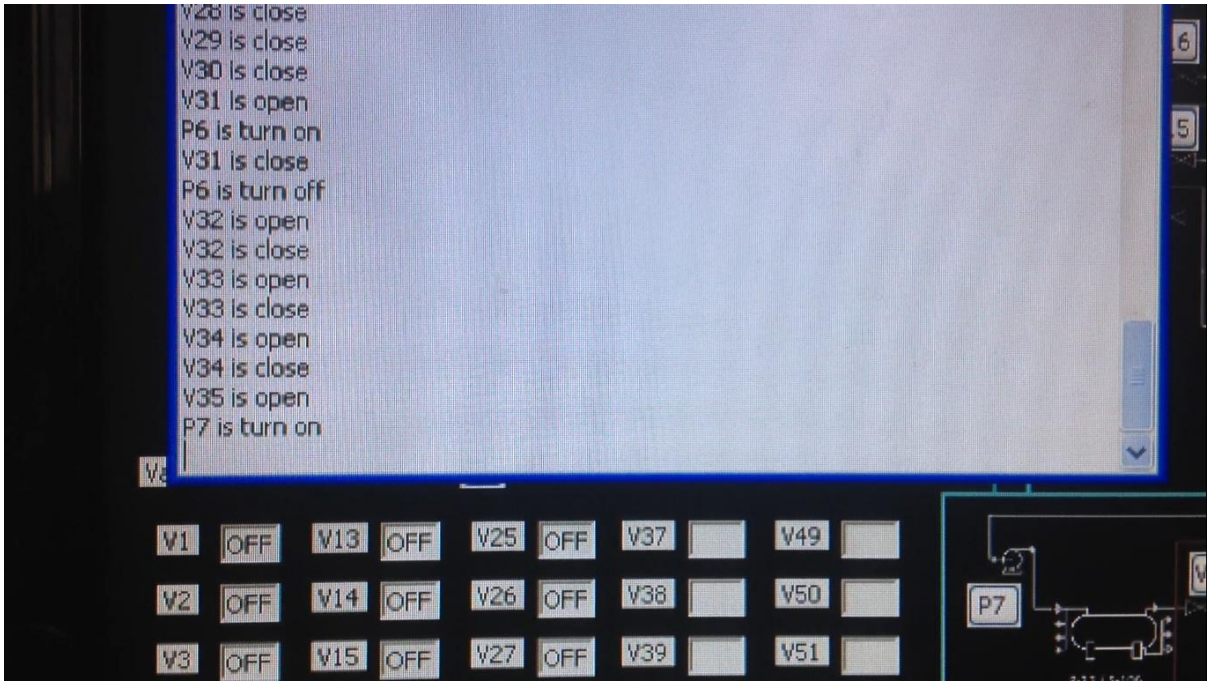
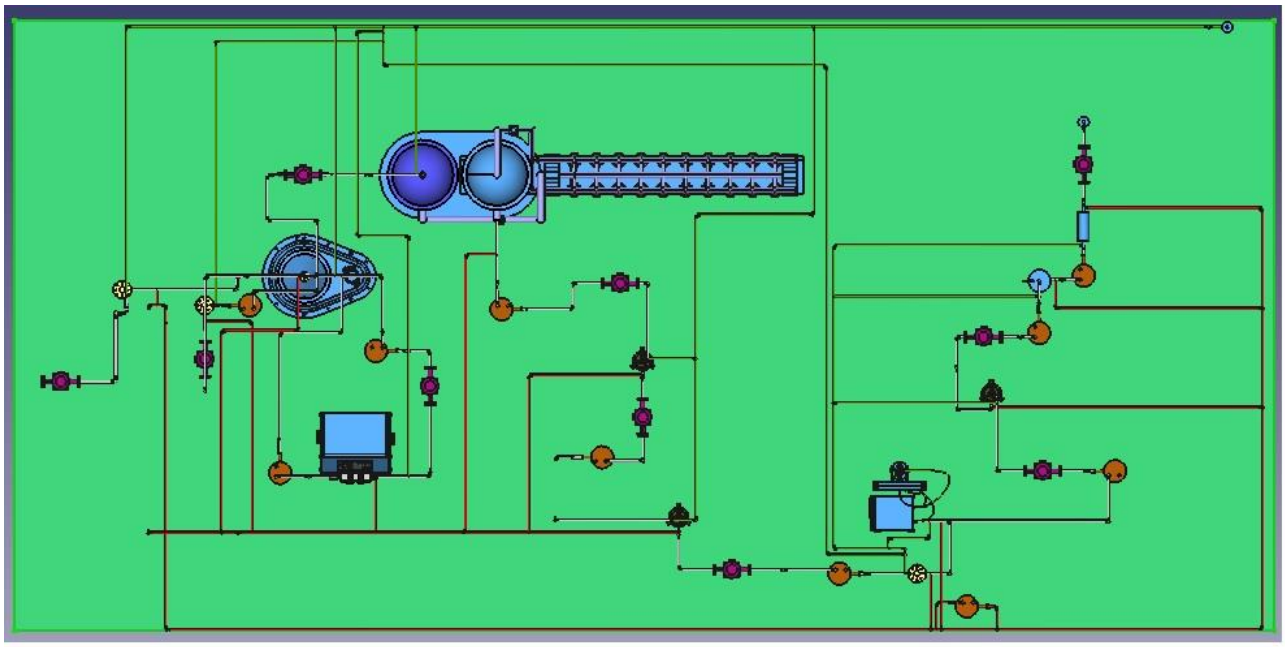


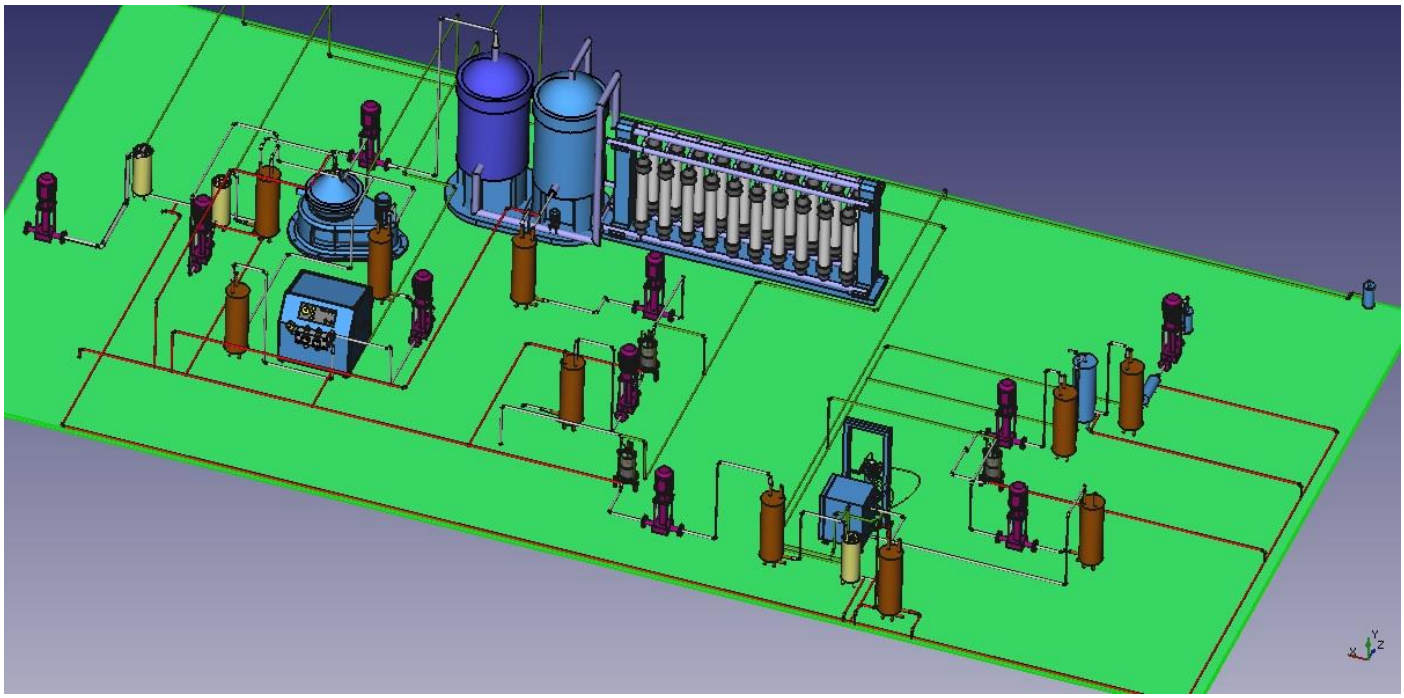
Figure 3.7: The test of the automation system by last step.

The system continues the step to finish at all the step of production and they automatically turned off, so the full automation is working and the test is succeed.

18 Integration of MEGBI-VPP based on Budget Plan for completing a minimal Prototype Plant

18.1 Overview





This version (minimal Prototype Plant) includes:

- smaller vessels

- simple disc stack centrifuge

- cost reduction option: vessel with chemical opening of cells instead of homogenizer

- disc stack centrifuge instead of ultracentifuge

- main new manufactoring: chromatographic columns (requires fine mechanical manufactoring facility)

18.2 Cost Planning November 15

Downstream Processing	# of mechnical pieces	average complexity\cost of part			price per piece	pieces needed	Costs
Disc Stack Centrifuge	20	$70			$1.400	1	$1.400
Homogenizer	10	$120			$1.200	1	$1.200
Chromatogr. Column (mech)	8	$50		 	$400	3	$1.200
Ultrafiltration Unit					$200	1	$200
Ultracentrifuge (used)		$70			$1.400	1	$1.400

Mixing/Storing tanks						$60	14	$840
							Total Devices Integration (piping, ...) 1/2 inch	$6.240 $1.500
Automation System								
Item	price per piece	# of pieces						
pump	$60	10						
electronic valve	$50	58						
	Total price	$3.500						
	Mechanical Parts USP	$0						
	Mechanical Parts DSP	$7.740						
	Automation System	$3.500						
	Total MEGBI-VPP Rest Budget Requirement	**$11.240**						

Alternative was:

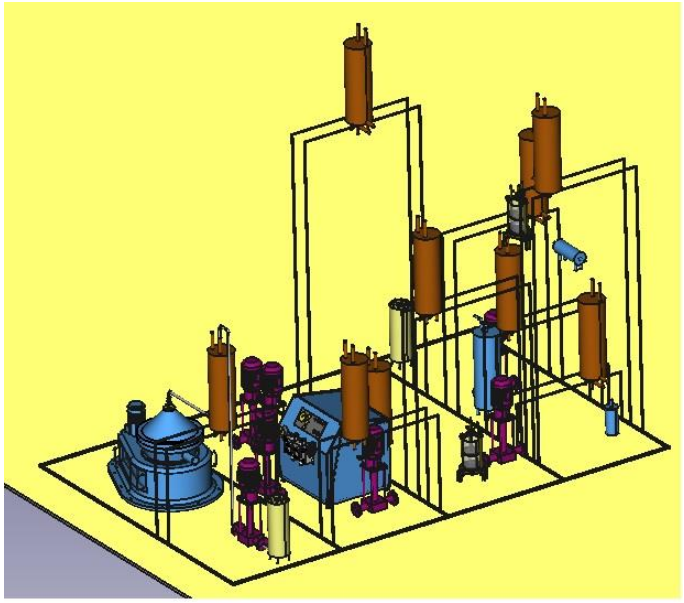
- Manufactoring of Devices and Integration. **Budget: 20.000 USD** (Material Costs: 10.000 USD, Personnel Cost: 4 months welder: 4000 USD, 4 months Eng.: 6000 USD)

Planned for Nov 15 - Feb 2016

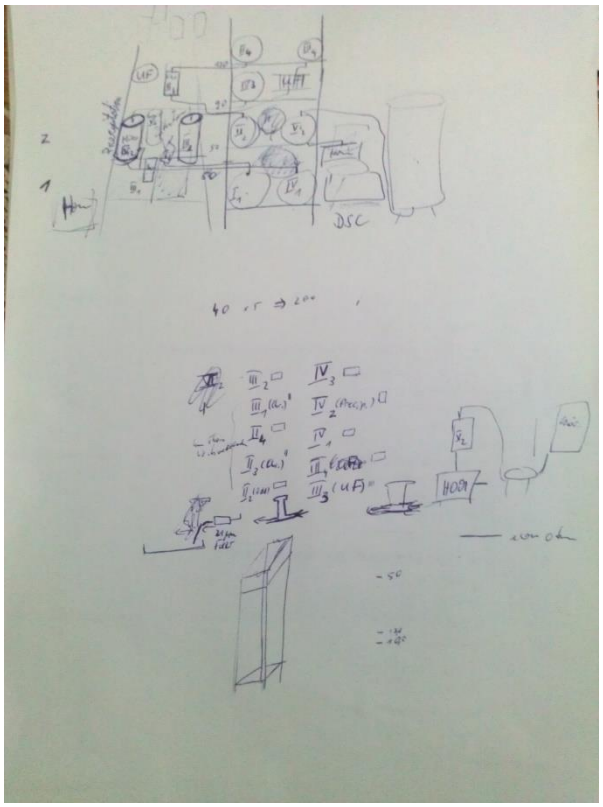
- Integration of Automation System to the whole plant (**Material Budget: 10 000 USD**),

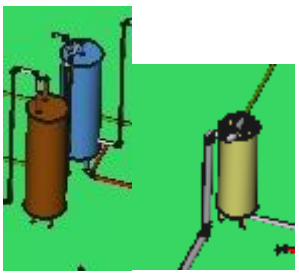
Planned for Mar-Aug 16 (as Master Thesis)

18.3 Manufactoring Planning of minimal system



18.3.1 Package 1: vessels for storing and mixing





Placing of storages based on flowdiagram

Costs: 1500$

18.3.2 Package 2: Chromatographic Columns, Disc Stack Centrifuge, Homogenizer

Costs: 1400$

18.3.3 Package 3: Pumps & Valves

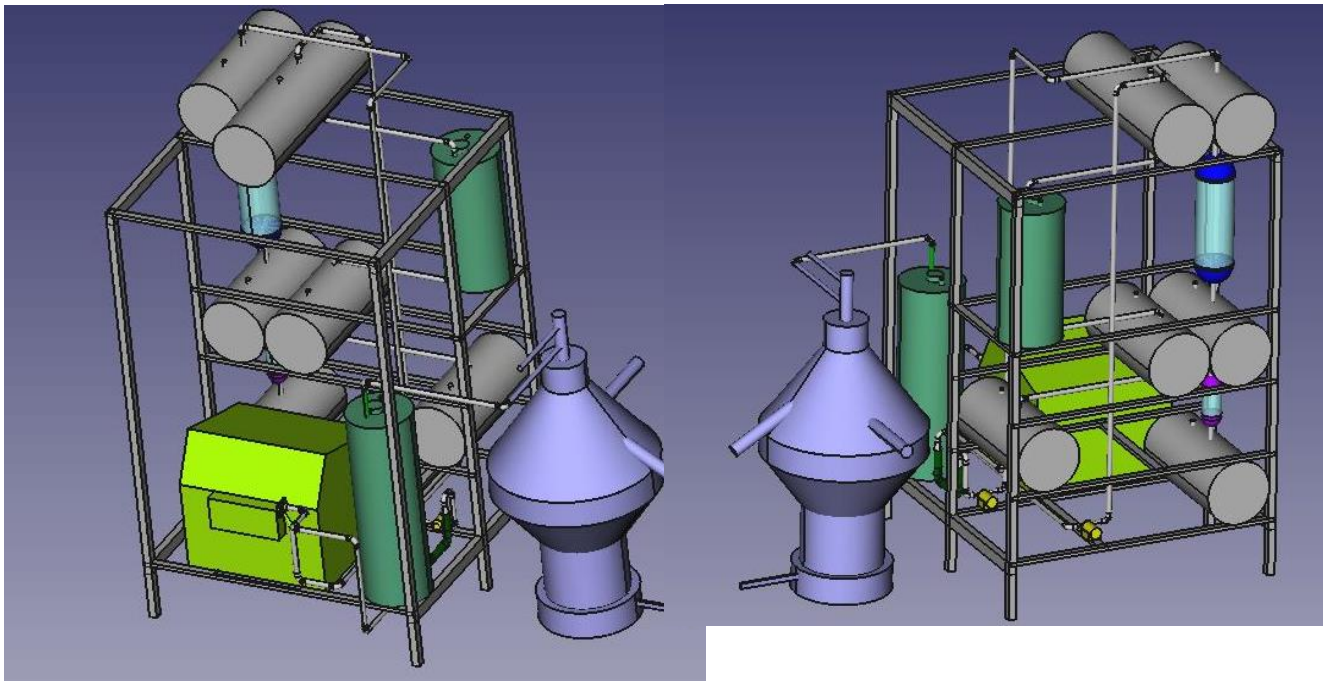
Costs: 3500$

18.3.4 Package 4: Piping

Costs: 1500$

18.4 Manufactored 24.12.-30.12.2015 (based on minimal system)

18.4.1 Design



18.4.2 Costs

1 sheet stainless steel 316: 1m x2m x 2mm -> about 150$

1 sheet stainless steel 304: 1m x2m x 2mm -> about 100$

stage material: 6m x 38 mm x 38 mm -> 35$

Material	Staff
storage & mixing tanks (stainless steel 316) and metal stage (stainless steel) -> about 850 $ 4 rolls (15$) 1 pump stainless steel 1,5 bar (55$)	Welder (Mohammad Qammah) for 7 days (all in 315 $)

There were total costs of 1230 $.

18.5 Still missing (to be manufactored/buyed in 2016 insha Allah)

piping

disc stack centrifuge

homogenizer

2 columns

Adaption of automation system

For manufactoring disc stack centrifuge and homoginizer a CNC machine is needed.

19 Annex

19.1 Annex 1a: How to install Python

- download python from www.python.org , we downloading python 3.5 version for windows 8

-install Python by run the program and continue the steps.

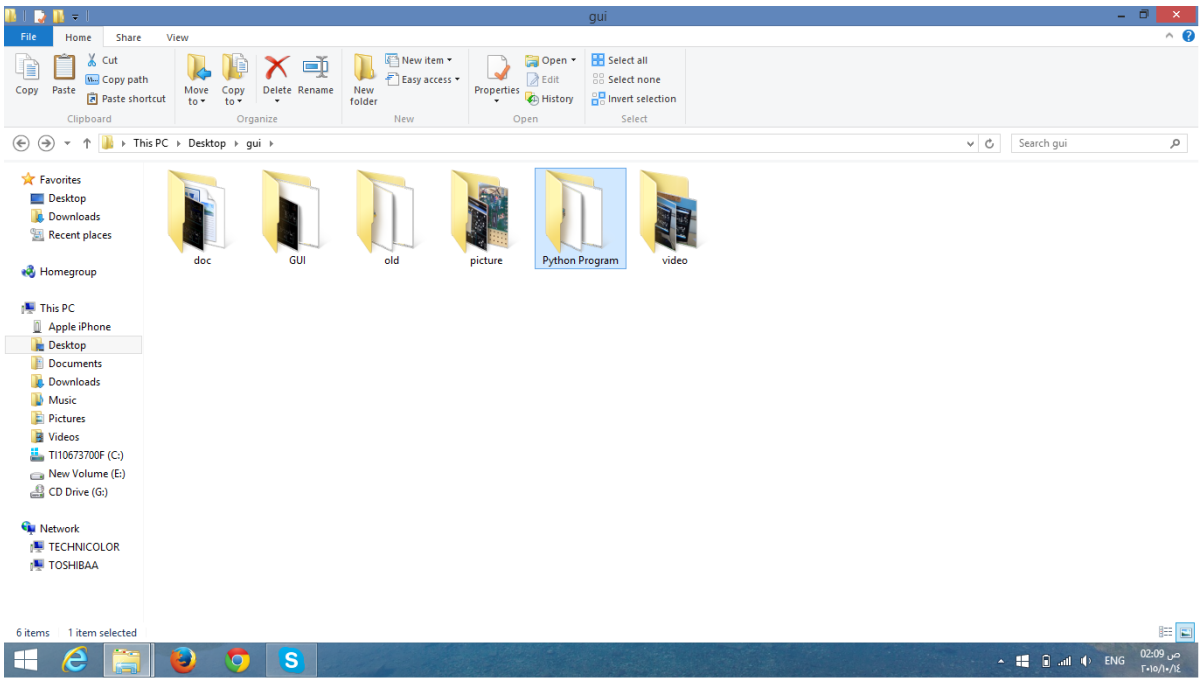
Needed a series of packages:

- Numpy-1.9.2
- Py2exe
- PyDDE
- wxPython-3.0.2.0

To install each package u should to open a command and write:

"C:\location of your python\Scripts\Pip install package name."

We have putted a CD with this document concerning:



19.2 Annex 1b: Connecting Veleman Board, Drivers

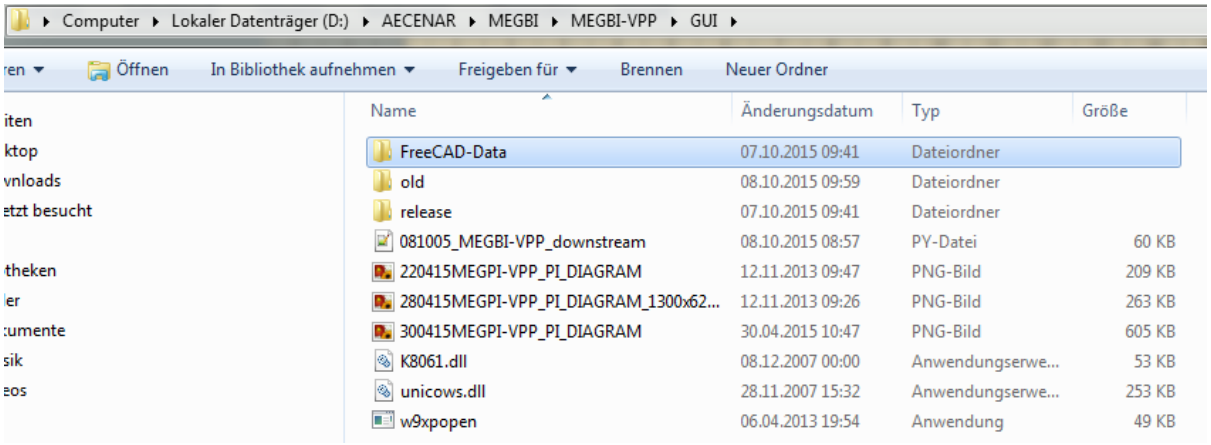
For the veleman board there are needed drivers. These drivers are only compatible to Windows XP, not to Windows 7. Following files are needed:

K8061.dll

unicows.dll

w9xpopen

The drivers must be in the same folder as the .py file.



19.3 Annex 2a: phython automation code only in maintance mode (without full automation)

```
##!/usr/bin/env python
## -*- coding: iso-8859-15 -*-
import sys
from tkinter import *
import random
import time
import wx
import thread



#**********************                    panel frame                     **********************
#*************************************************************************************************

BACKGROUND_IMAGENAME = "220415MEGPI-VPP_PI_DIAGRAM.PNG"


class MyBackgroundPanel(wx.Panel):

    def __init__(self, parent):
        wx.Panel.__init__(self, parent)
        self.bmp = wx.Bitmap(BACKGROUND_IMAGENAME)
        self.SetSize(self.bmp.GetSize())
        self.Bind(wx.EVT_PAINT, self.on_paint)


    def on_paint(self, event = None):
        dc = wx.BufferedPaintDC(self, self.bmp)

class MyFrame(wx.Frame):

    def __init__(self, parent = None, title = "MEGBI Vaccine Pilot Plant (MEGBI-VPP) Overview Upstream & Downstream Process"):

        self.testUSB                = True
        self.dll                    = None
        self.USBAdr0                = 0
        self.USBOpened              = False
        self.counterUSBBoards       = 3

        wx.Frame.__init__(self, parent, -1, title)

        panel = MyBackgroundPanel(self)

        LABELSTYLE = wx.BORDER_SUNKEN | wx.ST_NO_AUTORESIZE | wx.ALIGN_CENTER_HORIZONTAL


        menuFile = wx.Menu()
        menuFile.Append(1, "&About...")
        menuFile.AppendSeparator()
        menuFile.Append(2, "Exit")
        menuBar = wx.MenuBar()
        menuBar.Append(menuFile, "File")
        self.SetMenuBar(menuBar)
        self.CreateStatusBar()
        self.SetStatusText("Welcome to MEGPI Project!")
        self.Bind(wx.EVT_MENU, self.OnAbout, id=1)
        self.Bind(wx.EVT_MENU, self.OnQuit, id=2)
```

```
        # temperature sensors value

wx.StaticText(panel,-1," Temperature Value ",(100,12))
self.temp_Vaporizer_out = wx.StaticText(panel, size = (26, -1), pos = (200, 10), style = LABELSTYLE )



new_value = str(self.dll.ReadAnalogChannel(0,1))
self.temp_Vaporizer_out.SetLabel(new_value)
self.temp_Vaporizer_out.Refresh()


            #Valves

                #V1
self.button_V_1 = wx.Button(panel, -1, "V1", pos=(95,100),size=(20,20))
self.Bind(wx.EVT_BUTTON, self.V1, self.button_V_1)

                #V2
self.button_V_2 = wx.Button(panel, -1, "V2", pos=(150,90),size=(20,20))
self.Bind(wx.EVT_BUTTON, self.V2, self.button_V_2)
                #V3
self.button_V_3 = wx.Button(panel, -1, "V3", pos=(150,135),size=(20,20))
self.Bind(wx.EVT_BUTTON, self.V3, self.button_V_3)


                #V4
self.button_V_4 = wx.Button(panel, -1, "V4", pos=(150,265),size=(20,20))
self.Bind(wx.EVT_BUTTON, self.V4, self.button_V_4)

                #V5
self.button_V_5 = wx.Button(panel, -1, "V5", pos=(210,135),size=(20,20))
self.Bind(wx.EVT_BUTTON, self.V5, self.button_V_5)


                #V6
self.button_V_6 = wx.Button(panel, -1, "V6", pos=(215,185),size=(20,20))
self.Bind(wx.EVT_BUTTON, self.V6, self.button_V_6)

                #V7
self.button_V_7 = wx.Button(panel, -1, "V7", pos=(215,220),size=(20,20))
self.Bind(wx.EVT_BUTTON, self.V7, self.button_V_7)

                #V8
self.button_V_8 = wx.Button(panel, -1, "V8", pos=(282,180),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V8, self.button_V_8)

                #V9
self.button_V_9 = wx.Button(panel, -1, "V9", pos=(282,158),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V9, self.button_V_9)

                #V10
self.button_V_10 = wx.Button(panel, -1, "V10", pos=(322,110),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V10, self.button_V_10)

                #V11
self.button_V_11 = wx.Button(panel, -1, "V11", pos=(380,250),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V11, self.button_V_11)

                #V12
self.button_V_12 = wx.Button(panel, -1, "V12", pos=(413,195),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V12, self.button_V_12)

                #V13
self.button_V_13 = wx.Button(panel, -1, "V13", pos=(380,130),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V13, self.button_V_13)

                #V14
self.button_V_14 = wx.Button(panel, -1, "V14", pos=(410,100),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V14, self.button_V_14)
```

```
                #V15
self.button_V_15 = wx.Button(panel, -1, "V15", pos=(460,112),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V15, self.button_V_15)

                #V16
self.button_V_16 = wx.Button(panel, -1, "V16", pos=(460,73),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V16, self.button_V_16)

                #V17
self.button_V_17 = wx.Button(panel, -1, "V17", pos=(460,38),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V17, self.button_V_17)



                #V18
self.button_V_18 = wx.Button(panel, -1, "V18", pos=(522,18),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V18, self.button_V_18)

                #V19
self.button_V_19 = wx.Button(panel, -1, "V19", pos=(635,18),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V19, self.button_V_19)

                #V20
self.button_V_20 = wx.Button(panel, -1, "V20", pos=(635,72),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V20, self.button_V_20)

                #V21
self.button_V_21 = wx.Button(panel, -1, "V21", pos=(675,112),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V21, self.button_V_21)

                #V22
self.button_V_22 = wx.Button(panel, -1, "V22", pos=(635,155),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V22, self.button_V_22)

                #V23
self.button_V_23 = wx.Button(panel, -1, "V23", pos=(780,18),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V23, self.button_V_23)

                #V24
self.button_V_24 = wx.Button(panel, -1, "V24", pos=(770,120),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V24, self.button_V_24)

                #V25
self.button_V_25 = wx.Button(panel, -1, "V25", pos=(770,155),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V25, self.button_V_25)

                #V26
self.button_V_26 = wx.Button(panel, -1, "V26", pos=(870,155),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V26, self.button_V_26)


                #V27
self.button_V_27 = wx.Button(panel, -1, "V27", pos=(510,185),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V27, self.button_V_27)

                #V28
self.button_V_28 = wx.Button(panel, -1, "V28", pos=(620,185),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V28, self.button_V_28)

                #V29
self.button_V_29 = wx.Button(panel, -1, "V29", pos=(680,185),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V29, self.button_V_29)

                #V30
self.button_V_30 = wx.Button(panel, -1, "V30", pos=(620,240),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V30, self.button_V_30)

                #V31
self.button_V_31 = wx.Button(panel, -1, "V31", pos=(710,235),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V31, self.button_V_31)
```

```
                #V32
self.button_V_32 = wx.Button(panel, -1, "V32", pos=(950,182),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V32, self.button_V_32)

                #V33
self.button_V_33 = wx.Button(panel, -1, "V33", pos=(977,182),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V33, self.button_V_33)

                #V34
self.button_V_34 = wx.Button(panel, -1, "V34", pos=(1083,188),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V34, self.button_V_34)

                #V35
self.button_V_35 = wx.Button(panel, -1, "V35", pos=(1083,220),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V35, self.button_V_35)

                #V36
self.button_V_36 = wx.Button(panel, -1, "V36", pos=(1170,110),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V36, self.button_V_36)

                #V37
self.button_V_37 = wx.Button(panel, -1, "V37", pos=(1200,110),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V37, self.button_V_37)

                #V38
self.button_V_38 = wx.Button(panel, -1, "V38", pos=(1240,150),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V38, self.button_V_38)

                #V39
self.button_V_39 = wx.Button(panel, -1, "V39", pos=(1273,205),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V39, self.button_V_39)

                #V40
self.button_V_40 = wx.Button(panel, -1, "V40", pos=(483,314),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V40, self.button_V_40)


               #V41
self.button_V_41 = wx.Button(panel, -1, "V41", pos=(478,398),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V41, self.button_V_41)
               #V42
self.button_V_42 = wx.Button(panel, -1, "V42", pos=(540,314),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V42, self.button_V_42)
               #V43
self.button_V_43 = wx.Button(panel, -1, "V43", pos=(548,398),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V43, self.button_V_43)
               #V44
self.button_V_44 = wx.Button(panel, -1, "V44", pos=(580,300),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V44, self.button_V_44)
               #V45
self.button_V_45 = wx.Button(panel, -1, "V45", pos=(620,314),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V45, self.button_V_45)
               #V46
self.button_V_46 = wx.Button(panel, -1, "V46", pos=(760,440),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V46, self.button_V_46)
               #V47
self.button_V_47 = wx.Button(panel, -1, "V47", pos=(760,400),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V47, self.button_V_47)
               #V48
self.button_V_48 = wx.Button(panel, -1, "V48", pos=(783,375),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V48, self.button_V_48)
               #V49
self.button_V_49 = wx.Button(panel, -1, "V49", pos=(880,428),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V49, self.button_V_49)
               #V50
self.button_V_50 = wx.Button(panel, -1, "V50", pos=(970,377),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V50, self.button_V_50)
               #V51
self.button_V_51 = wx.Button(panel, -1, "V51", pos=(963,450),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V51, self.button_V_51)
               #V52
self.button_V_52 = wx.Button(panel, -1, "V52", pos=(1005,470),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V52, self.button_V_52)
```

```
                #V52
self.button_V_52 = wx.Button(panel, -1, "V52", pos=(1005,470),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V52, self.button_V_52)
                #V53
self.button_V_53 = wx.Button(panel, -1, "V53", pos=(1030,395),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V53, self.button_V_53)
                #V54
self.button_V_54 = wx.Button(panel, -1, "V54", pos=(1080,395),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V54, self.button_V_54)
                #V55
self.button_V_55 = wx.Button(panel, -1, "V55", pos=(1118,377),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V55, self.button_V_55)
                #V56
self.button_V_56 = wx.Button(panel, -1, "V56", pos=(1158,468),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V56, self.button_V_56)
                #V57
self.button_V_57 = wx.Button(panel, -1, "V57", pos=(1190,420),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V57, self.button_V_57)
                #V58
self.button_V_58 = wx.Button(panel, -1, "V58", pos=(710,580),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V58, self.button_V_58)


            #Pumps

                #P1
self.button_P_1 = wx.Button(panel, -1, "P1", pos=(150,180),size=(20,20))
self.Bind(wx.EVT_BUTTON, self.P1, self.button_P_1)
                #P2
self.button_P_2 = wx.Button(panel, -1, "P2", pos=(150,220),size=(20,20))
self.Bind(wx.EVT_BUTTON, self.P2, self.button_P_2)
                #P3
self.button_P_3 = wx.Button(panel, -1, "P3", pos=(510,40),size=(20,20))
self.Bind(wx.EVT_BUTTON, self.P3, self.button_P_3)
                #P4
self.button_P_4 = wx.Button(panel, -1, "P4", pos=(880,18),size=(20,20))
self.Bind(wx.EVT_BUTTON, self.P4, self.button_P_4)

                #P5
self.button_P_5 = wx.Button(panel, -1, "P5", pos=(730,187),size=(20,20))
self.Bind(wx.EVT_BUTTON, self.P5, self.button_P_5)

                #P6
self.button_P_6 = wx.Button(panel, -1, "P6", pos=(1083,130),size=(20,20))
self.Bind(wx.EVT_BUTTON, self.P6, self.button_P_6)

                #P7
self.button_P_7 = wx.Button(panel, -1, "P7", pos=(380,328),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.P7, self.button_P_7)

                #P8
self.button_P_8 = wx.Button(panel, -1, "P8", pos=(710,423),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.P8, self.button_P_8)

                #P9
self.button_P_9 = wx.Button(panel, -1, "P9", pos=(900,380),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.P9, self.button_P_9)

                #P10
self.button_P_10 = wx.Button(panel, -1, "P10", pos=(1200,380),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.P10, self.button_P_10)
```

```
monitoring valve status


    #Text column 1
wx.StaticText(panel,20," Valve Selected : ",(10,270))
wx.StaticText(panel,-1," V1 ",(18,300))
wx.StaticText(panel,-1," V2 ",(18,326))
wx.StaticText(panel,-1," V3 ",(18,352))
wx.StaticText(panel,-1," V4 ",(18,378))
wx.StaticText(panel,-1," V5 ",(18,402))
wx.StaticText(panel,-1," V6 ",(18,428))
wx.StaticText(panel,-1," V7 ",(18,454))
wx.StaticText(panel,-1," V8 ",(18,480))
wx.StaticText(panel,-1," V9 ",(18,506))
wx.StaticText(panel,-1," V10 ",(18,532))
wx.StaticText(panel,-1," V11 ",(18,558))
wx.StaticText(panel,-1," V12 ",(18,584))

    #Text column 2
wx.StaticText(panel,-1," V13 ",(85,300))
wx.StaticText(panel,-1," V14 ",(85,326))
wx.StaticText(panel,-1," V15 ",(85,352))
wx.StaticText(panel,-1," V16 ",(85,378))
wx.StaticText(panel,-1," V17 ",(85,402))
wx.StaticText(panel,-1," V18 ",(85,428))
wx.StaticText(panel,-1," V19 ",(85,454))
wx.StaticText(panel,-1," V20 ",(85,480))
wx.StaticText(panel,-1," V21 ",(85,506))
wx.StaticText(panel,-1," V22 ",(85,532))
wx.StaticText(panel,-1," V23 ",(85,558))
wx.StaticText(panel,-1," V24 ",(85,584))

 #Text column 3
wx.StaticText(panel,-1," V25 ",(155,300))
wx.StaticText(panel,-1," V26 ",(155,326))
wx.StaticText(panel,-1," V27 ",(155,352))
wx.StaticText(panel,-1," V28 ",(155,378))
wx.StaticText(panel,-1," V29 ",(155,402))
wx.StaticText(panel,-1," V30 ",(155,428))
wx.StaticText(panel,-1," V31 ",(155,454))
wx.StaticText(panel,-1," V32 ",(155,480))
wx.StaticText(panel,-1," V33 ",(155,506))
wx.StaticText(panel,-1," V34 ",(155,532))
wx.StaticText(panel,-1," V35 ",(155,558))
wx.StaticText(panel,-1," V36 ",(155,584))

#Text column 4
wx.StaticText(panel,-1," V37 ",(223,300))
wx.StaticText(panel,-1," V38 ",(223,326))
wx.StaticText(panel,-1," V39 ",(223,352))
wx.StaticText(panel,-1," V40 ",(223,378))
wx.StaticText(panel,-1," V41 ",(223,402))
wx.StaticText(panel,-1," V42 ",(223,428))
wx.StaticText(panel,-1," V43 ",(223,454))
wx.StaticText(panel,-1," V44 ",(223,480))
wx.StaticText(panel,-1," V45 ",(223,506))
wx.StaticText(panel,-1," V46 ",(223,532))
wx.StaticText(panel,-1," V47 ",(223,558))
wx.StaticText(panel,-1," V48 ",(223,584))

#Text column 5
wx.StaticText(panel,-1," V49 ",(293,300))
wx.StaticText(panel,-1," V50 ",(293,326))
wx.StaticText(panel,-1," V51 ",(293,352))
wx.StaticText(panel,-1," V52 ",(293,378))
wx.StaticText(panel,-1," V53 ",(293,402))
wx.StaticText(panel,-1," V54 ",(293,428))
wx.StaticText(panel,-1," V55 ",(293,454))
wx.StaticText(panel,-1," V56 ",(293,480))
wx.StaticText(panel,-1," V57 ",(293,506))
wx.StaticText(panel,-1," V58 ",(293,532))
```

```
    #Label  Column 1
self.Valve1 = wx.StaticText(panel, size = (26, -1), pos = (45, 300), style = LABELSTYLE)
self.Valve2 = wx.StaticText(panel, size = (26, -1), pos = (45, 326), style = LABELSTYLE)
self.Valve3 = wx.StaticText(panel, size = (26, -1), pos = (45, 352), style = LABELSTYLE)
self.Valve4 = wx.StaticText(panel, size = (26, -1), pos = (45, 378), style = LABELSTYLE)
self.Valve5 = wx.StaticText(panel, size = (26, -1), pos = (45, 402), style = LABELSTYLE)
self.Valve6 = wx.StaticText(panel, size = (26, -1), pos = (45, 428), style = LABELSTYLE)
self.Valve7 = wx.StaticText(panel, size = (26, -1), pos = (45, 454), style = LABELSTYLE)
self.Valve8 = wx.StaticText(panel, size = (26, -1), pos = (45, 480), style = LABELSTYLE)
self.Valve9 = wx.StaticText(panel, size = (26, -1), pos = (45, 506), style = LABELSTYLE)
self.Valve10 = wx.StaticText(panel, size = (26, -1), pos = (45, 532), style = LABELSTYLE)
self.Valve11 = wx.StaticText(panel, size = (26, -1), pos = (45, 558), style = LABELSTYLE)
self.Valve12 = wx.StaticText(panel, size = (26, -1), pos = (45, 584), style = LABELSTYLE)



    #Label column 2
self.Valve13 = wx.StaticText(panel, size = (26, -1), pos = (115, 300), style = LABELSTYLE)
self.Valve14 = wx.StaticText(panel, size = (26, -1), pos = (115, 326), style = LABELSTYLE)
self.Valve15 = wx.StaticText(panel, size = (26, -1), pos = (115, 352), style = LABELSTYLE)
self.Valve16 = wx.StaticText(panel, size = (26, -1), pos = (115, 378), style = LABELSTYLE)
self.Valve17 = wx.StaticText(panel, size = (26, -1), pos = (115, 402), style = LABELSTYLE)
self.Valve18 = wx.StaticText(panel, size = (26, -1), pos = (115, 428), style = LABELSTYLE)
self.Valve19 = wx.StaticText(panel, size = (26, -1), pos = (115, 454), style = LABELSTYLE)
self.Valve20 = wx.StaticText(panel, size = (26, -1), pos = (115, 480), style = LABELSTYLE)
self.Valve21 = wx.StaticText(panel, size = (26, -1), pos = (115, 506), style = LABELSTYLE)
self.Valve22 = wx.StaticText(panel, size = (26, -1), pos = (115, 532), style = LABELSTYLE)
self.Valve23 = wx.StaticText(panel, size = (26, -1), pos = (115, 558), style = LABELSTYLE)
self.Valve24 = wx.StaticText(panel, size = (26, -1), pos = (115, 584), style = LABELSTYLE)

 #Label column 3

self.Valve25 = wx.StaticText(panel, size = (26, -1), pos = (185, 300), style = LABELSTYLE)
self.Valve26 = wx.StaticText(panel, size = (26, -1), pos = (185, 326), style = LABELSTYLE)
self.Valve27 = wx.StaticText(panel, size = (26, -1), pos = (185, 352), style = LABELSTYLE)
self.Valve28 = wx.StaticText(panel, size = (26, -1), pos = (185, 378), style = LABELSTYLE)
self.Valve29 = wx.StaticText(panel, size = (26, -1), pos = (185, 402), style = LABELSTYLE)
self.Valve30 = wx.StaticText(panel, size = (26, -1), pos = (185, 428), style = LABELSTYLE)
self.Valve31 = wx.StaticText(panel, size = (26, -1), pos = (185, 454), style = LABELSTYLE)
self.Valve32 = wx.StaticText(panel, size = (26, -1), pos = (185, 480), style = LABELSTYLE)
self.Valve33 = wx.StaticText(panel, size = (26, -1), pos = (185, 506), style = LABELSTYLE)
self.Valve34 = wx.StaticText(panel, size = (26, -1), pos = (185, 532), style = LABELSTYLE)
self.Valve35 = wx.StaticText(panel, size = (26, -1), pos = (185, 558), style = LABELSTYLE)
self.Valve36 = wx.StaticText(panel, size = (26, -1), pos = (185, 584), style = LABELSTYLE)

#Label column 4

self.Valve37 = wx.StaticText(panel, size = (26, -1), pos = (253, 300), style = LABELSTYLE)
self.Valve38 = wx.StaticText(panel, size = (26, -1), pos = (253, 326), style = LABELSTYLE)
self.Valve39 = wx.StaticText(panel, size = (26, -1), pos = (253, 352), style = LABELSTYLE)
self.Valve40 = wx.StaticText(panel, size = (26, -1), pos = (253, 378), style = LABELSTYLE)
self.Valve41 = wx.StaticText(panel, size = (26, -1), pos = (253, 402), style = LABELSTYLE)
self.Valve42 = wx.StaticText(panel, size = (26, -1), pos = (253, 428), style = LABELSTYLE)
self.Valve43 = wx.StaticText(panel, size = (26, -1), pos = (253, 454), style = LABELSTYLE)
self.Valve44 = wx.StaticText(panel, size = (26, -1), pos = (253, 480), style = LABELSTYLE)
self.Valve45 = wx.StaticText(panel, size = (26, -1), pos = (253, 506), style = LABELSTYLE)
self.Valve46 = wx.StaticText(panel, size = (26, -1), pos = (253, 532), style = LABELSTYLE)
self.Valve47 = wx.StaticText(panel, size = (26, -1), pos = (253, 558), style = LABELSTYLE)
self.Valve48 = wx.StaticText(panel, size = (26, -1), pos = (253, 584), style = LABELSTYLE)
#Label column 5

self.Valve49 = wx.StaticText(panel, size = (26, -1), pos = (323, 300), style = LABELSTYLE)
self.Valve50 = wx.StaticText(panel, size = (26, -1), pos = (323, 326), style = LABELSTYLE)
self.Valve51 = wx.StaticText(panel, size = (26, -1), pos = (323, 352), style = LABELSTYLE)
self.Valve52 = wx.StaticText(panel, size = (26, -1), pos = (323, 378), style = LABELSTYLE)
self.Valve53 = wx.StaticText(panel, size = (26, -1), pos = (323, 402), style = LABELSTYLE)
self.Valve54 = wx.StaticText(panel, size = (26, -1), pos = (323, 428), style = LABELSTYLE)
self.Valve55 = wx.StaticText(panel, size = (26, -1), pos = (323, 454), style = LABELSTYLE)
self.Valve56 = wx.StaticText(panel, size = (26, -1), pos = (323, 480), style = LABELSTYLE)
self.Valve57 = wx.StaticText(panel, size = (26, -1), pos = (323, 506), style = LABELSTYLE)
self.Valve58 = wx.StaticText(panel, size = (26, -1), pos = (323, 532), style = LABELSTYLE)


self.P1 = wx.StaticText(panel, size = (26, -1), pos = (60, 10), style = LABELSTYLE)
self.P2 = wx.StaticText(panel, size = (26, -1), pos = (60, 40), style = LABELSTYLE)
wx.StaticText(panel,-1," P1 ",(20,10))
wx.StaticText(panel,-1," P2 ",(20,40))
```

```
#**********************                Run USB System                      **********************
#*****************************************************************************************************
    def OpenUSBBoardThread(self):
        self.dll = windll.K8061
        i = self.counterUSBBoards
        for doit in range(0,i+1):
            try:
                self.dll.OpenDevice()
                self.USBOpened = True
# debug info
                print ('USB Board is now connected!')
#end debug info
            except:
                txt = ('Please Check USB Board connection')
                print ('txt')
                return


############################       Menu Bar              ##########################
#*************************************************************************************
    def OnAbout(self, event):
        wx.MessageBox("This is a Screen controller of MEGPI Project",
                      "Welcome to my python", wx.OK | wx.ICON_INFORMATION, self)
    def OnQuit(self, event):
        self.Close()
    def on_timer(self, event = None):
        new_value = str(windll.K8061.ReadAnalogChannel(0,1))

#**********************                      Define Valvue                     ********************
#*****************************************************************************************************
    def V1(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'

# open the USB board

            if  self.Valve1.GetLabel()==valve_status_OFF:
                self.Valve1.SetLabel(valve_status_ON)
                self.OpenUSBBoardThread()
                self.dll.SetDigitalChannel(0,1)
                wx.MessageBox("V1 is open ", "Open", wx.OK|wx.ICON_INFORMATION)
                self.timer = wx.Timer()
                self.timer.Bind(wx.EVT_TIMER, self.on_timer)
                self.timer.Start(0)

            else:
                self.Valve1.SetLabel(valve_status_OFF)
                time.sleep(2)
                self.dll.ClearDigitalChannel(0,1)
                print 'Digital Channel Cleared, V1 turn off'

    def V2(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'

            if self.Valve2.GetLabel()==valve_status_ON:
                self.Valve2.SetLabel(valve_status_OFF)

            else:
                self.Valve2.SetLabel(valve_status_ON)
```

```
    def V3(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve3.GetLabel()==valve_status_ON:
                self.Valve3.SetLabel(valve_status_OFF)
            else:
                self.Valve3.SetLabel(valve_status_ON)

    def V4(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve4.GetLabel()==valve_status_ON:
                self.Valve4.SetLabel(valve_status_OFF)
            else:
               self.Valve4.SetLabel(valve_status_ON)


    def V5(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve5.GetLabel()==valve_status_ON:
                self.Valve5.SetLabel(valve_status_OFF)
            else:
                self.Valve5.SetLabel(valve_status_ON)

    def V6(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve6.GetLabel()==valve_status_ON:
                self.Valve6.SetLabel(valve_status_OFF)
            else:
                self.Valve6.SetLabel(valve_status_ON)

def V7(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'
        if self.Valve7.GetLabel()==valve_status_ON:
            self.Valve7.SetLabel(valve_status_OFF)
        else:
            self.Valve7.SetLabel(valve_status_ON)
```

```
    def V8(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve8.GetLabel()==valve_status_ON:
                self.Valve8.SetLabel(valve_status_OFF)
            else:
                self.Valve8.SetLabel(valve_status_ON)

    def V9(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve9.GetLabel()==valve_status_ON:
                self.Valve9.SetLabel(valve_status_OFF)
            else:
                self.Valve9.SetLabel(valve_status_ON)

    def V10(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve10.GetLabel()==valve_status_ON:
                self.Valve10.SetLabel(valve_status_OFF)
            else:
                self.Valve10.SetLabel(valve_status_ON)

    def V11(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve11.GetLabel()==valve_status_ON:
                self.Valve11.SetLabel(valve_status_OFF)

            else:
                self.Valve11.SetLabel(valve_status_ON)

     def V12(self, event):
             valve_status_ON = 'ON'
             valve_status_OFF = 'OFF'
             if self.Valve12.GetLabel()==valve_status_ON:
                 self.Valve12.SetLabel(valve_status_OFF)
             else:
                 self.Valve12.SetLabel(valve_status_ON)

     def V13(self, event):
             valve_status_ON = 'ON'
             valve_status_OFF = 'OFF'
             if self.Valve13.GetLabel()==valve_status_ON:
                 self.Valve13.SetLabel(valve_status_OFF)
             else:
                 self.Valve13.SetLabel(valve_status_ON)

     def V14(self, event):
             valve_status_ON = 'ON'
             valve_status_OFF = 'OFF'
             if self.Valve14.GetLabel()==valve_status_ON:
                 self.Valve14.SetLabel(valve_status_OFF)
             else:
                 self.Valve14.SetLabel(valve_status_ON)

     def V15(self, event):
             valve_status_ON = 'ON'
             valve_status_OFF = 'OFF'
             if self.Valve15.GetLabel()==valve_status_ON:
                 self.Valve15.SetLabel(valve_status_OFF)
             else:
                 self.Valve15.SetLabel(valve_status_ON)

    def V16(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve16.GetLabel()==valve_status_ON:
                self.Valve16.SetLabel(valve_status_OFF)
            else:
                self.Valve16.SetLabel(valve_status_ON)
```

```
    def V17(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve17.GetLabel()==valve_status_ON:
                self.Valve17.SetLabel(valve_status_OFF)
            else:
                self.Valve17.SetLabel(valve_status_ON)

    def V18(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve18.GetLabel()==valve_status_ON:
                self.Valve18.SetLabel(valve_status_OFF)
            else:
                self.Valve18.SetLabel(valve_status_ON)

    def V19(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve19.GetLabel()==valve_status_ON:
                self.Valve19.SetLabel(valve_status_OFF)
            else:
                self.Valve19.SetLabel(valve_status_ON)

    def V20(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve20.GetLabel()==valve_status_ON:
                self.Valve20.SetLabel(valve_status_OFF)
            else:
                self.Valve20.SetLabel(valve_status_ON)

    def V21(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve21.GetLabel()==valve_status_ON:
                self.Valve21.SetLabel(valve_status_OFF)
            else:
                self.Valve21.SetLabel(valve_status_ON)

    def V22(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve22.GetLabel()==valve_status_ON:
                self.Valve22.SetLabel(valve_status_OFF)
            else:
                self.Valve22.SetLabel(valve_status_ON)

    def V23(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve23.GetLabel()==valve_status_ON:
                self.Valve23.SetLabel(valve_status_OFF)
            else:
                self.Valve23.SetLabel(valve_status_ON)

    def V24(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve24.GetLabel()==valve_status_ON:
                self.Valve24.SetLabel(valve_status_OFF)
            else:
                self.Valve24.SetLabel(valve_status_ON)

    def V25(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve25.GetLabel()==valve_status_ON:
                self.Valve25.SetLabel(valve_status_OFF)
            else:
                self.Valve25.SetLabel(valve_status_ON)
```

```
    def V26(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve26.GetLabel()==valve_status_ON:
                self.Valve26.SetLabel(valve_status_OFF)
            else:
                self.Valve26.SetLabel(valve_status_ON)

    def V27(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve27.GetLabel()==valve_status_ON:
                self.Valve27.SetLabel(valve_status_OFF)
            else:
                self.Valve27.SetLabel(valve_status_ON)

    def V28(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve28.GetLabel()==valve_status_ON:
                self.Valve28.SetLabel(valve_status_OFF)
            else:
                self.Valve28.SetLabel(valve_status_ON)

    def V29(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'

            if self.Valve29.GetLabel()==valve_status_ON:
                self.Valve29.SetLabel(valve_status_OFF)
            else:
                self.Valve29.SetLabel(valve_status_ON)
    def V30(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve30.GetLabel()==valve_status_ON:
                self.Valve30.SetLabel(valve_status_OFF)
            else:
                self.Valve30.SetLabel(valve_status_ON)

    def V31(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve31.GetLabel()==valve_status_ON:
                self.Valve31.SetLabel(valve_status_OFF)
            else:
                self.Valve31.SetLabel(valve_status_ON)

    def V32(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve32.GetLabel()==valve_status_ON:
                self.Valve32.SetLabel(valve_status_OFF)
            else:
                self.Valve32.SetLabel(valve_status_ON)

    def V33(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve33.GetLabel()==valve_status_ON:
                self.Valve33.SetLabel(valve_status_OFF)
            else:
                self.Valve33.SetLabel(valve_status_ON)

    def V34(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'

            if self.Valve34.GetLabel()==valve_status_ON:
                self.Valve34.SetLabel(valve_status_OFF)

            else:
                self.Valve34.SetLabel(valve_status_ON)
```

```
    def V35(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve35.GetLabel()==valve_status_ON:
                self.Valve35.SetLabel(valve_status_OFF)
            else:
                self.Valve35.SetLabel(valve_status_ON)

    def V36(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve36.GetLabel()==valve_status_ON:
                self.Valve36.SetLabel(valve_status_OFF)
            else:
                self.Valve36.SetLabel(valve_status_ON)

    def V37(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve37.GetLabel()==valve_status_ON:
                self.Valve37.SetLabel(valve_status_OFF)
            else:
                self.Valve37.SetLabel(valve_status_ON)

    def V38(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve38.GetLabel()==valve_status_ON:
                self.Valve38.SetLabel(valve_status_OFF)
            else:
                self.Valve38.SetLabel(valve_status_ON)

    def V39(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve39.GetLabel()==valve_status_ON:
                self.Valve39.SetLabel(valve_status_OFF)
            else:
                self.Valve39.SetLabel(valve_status_ON)

    def V40(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve40.GetLabel()==valve_status_ON:
                self.Valve40.SetLabel(valve_status_OFF)
            else:
                self.Valve40.SetLabel(valve_status_ON)

    def V41(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve41.GetLabel()==valve_status_ON:
                self.Valve41.SetLabel(valve_status_OFF)
            else:
                self.Valve41.SetLabel(valve_status_ON)

    def V42(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve42.GetLabel()==valve_status_ON:
                self.Valve42.SetLabel(valve_status_OFF)
            else:
                self.Valve42.SetLabel(valve_status_ON)

    def V43(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'

            if self.Valve43.GetLabel()==valve_status_ON:
                self.Valve43.SetLabel(valve_status_OFF)

            else:
                self.Valve43.SetLabel(valve_status_ON)
```

```
def V44(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'
        if self.Valve44.GetLabel()==valve_status_ON:
            self.Valve44.SetLabel(valve_status_OFF)
        else:
            self.Valve44.SetLabel(valve_status_ON)

def V45(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'
        if self.Valve45.GetLabel()==valve_status_ON:
            self.Valve45.SetLabel(valve_status_OFF)
        else:
            self.Valve45.SetLabel(valve_status_ON)

def V46(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'
        if self.Valve46.GetLabel()==valve_status_ON:
            self.Valve46.SetLabel(valve_status_OFF)
        else:
            self.Valve46.SetLabel(valve_status_ON)

def V47(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'
        if self.Valve47.GetLabel()==valve_status_ON:
            self.Valve47.SetLabel(valve_status_OFF)
        else:
            self.Valve47.SetLabel(valve_status_ON)

def V48(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'
        if self.Valve48.GetLabel()==valve_status_ON:
            self.Valve48.SetLabel(valve_status_OFF)
        else:
            self.Valve48.SetLabel(valve_status_ON)

def V49(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'
        if self.Valve49.GetLabel()==valve_status_ON:
            self.Valve49.SetLabel(valve_status_OFF)
        else:
            self.Valve49.SetLabel(valve_status_ON)

def V50(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'
        if self.Valve50.GetLabel()==valve_status_ON:
            self.Valve50.SetLabel(valve_status_OFF)
        else:
            self.Valve50.SetLabel(valve_status_ON)

def V51(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'
        if self.Valve51.GetLabel()==valve_status_ON:
            self.Valve51.SetLabel(valve_status_OFF)
        else:
            self.Valve51.SetLabel(valve_status_ON)

 def V52(self, event):
         valve_status_ON = 'ON'
         valve_status_OFF = 'OFF'

         if self.Valve52.GetLabel()==valve_status_ON:
             self.Valve52.SetLabel(valve_status_OFF)

         else:
             self.Valve52.SetLabel(valve_status_ON)
```

```
    def V53(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve53.GetLabel()==valve_status_ON:
                self.Valve53.SetLabel(valve_status_OFF)
            else:
                self.Valve53.SetLabel(valve_status_ON)

    def V54(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve54.GetLabel()==valve_status_ON:
                self.Valve54.SetLabel(valve_status_OFF)
            else:
                self.Valve54.SetLabel(valve_status_ON)

    def V55(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve55.GetLabel()==valve_status_ON:
                self.Valve55.SetLabel(valve_status_OFF)
            else:
                self.Valve55.SetLabel(valve_status_ON)

    def V56(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve56.GetLabel()==valve_status_ON:
                self.Valve56.SetLabel(valve_status_OFF)
            else:
                self.Valve56.SetLabel(valve_status_ON)

    def V57(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve57.GetLabel()==valve_status_ON:
                self.Valve57.SetLabel(valve_status_OFF)
            else:
                self.Valve57.SetLabel(valve_status_ON)

    def V58(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'
            if self.Valve58.GetLabel()==valve_status_ON:
                self.Valve58.SetLabel(valve_status_OFF)
            else:
                self.Valve58.SetLabel(valve_status_ON)
#********************                        Define Pumps                        ********************
#*************************************************************************************************

    def P1 (self,event):
        wx.MessageBox("P3 is Open ","Pump", wx.OK|wx.ICON_INFORMATION)
    def P2 (self,event):
        wx.MessageBox("P3 is Open ","Pump", wx.OK|wx.ICON_INFORMATION)
    def P3 (self,event):
        wx.MessageBox("P3 is Open ","Pump", wx.OK|wx.ICON_INFORMATION)
    def P4 (self,event):
        wx.MessageBox("P4 is Open ","Pump", wx.OK|wx.ICON_INFORMATION)

    def P5 (self,event):
        wx.MessageBox("P5 is Open ","Pump", wx.OK|wx.ICON_INFORMATION)

    def P6 (self,event):
        wx.MessageBox("P6 is Open ","Pump", wx.OK|wx.ICON_INFORMATION)

    def P7 (self,event):
        wx.MessageBox("P7 is Open ","Pump", wx.OK|wx.ICON_INFORMATION)

    def P8 (self,event):
        wx.MessageBox("P8 is Open ","Pump", wx.OK|wx.ICON_INFORMATION)

    def P9 (self,event):
        wx.MessageBox("P9 is Open ","Pump", wx.OK|wx.ICON_INFORMATION)

    def P10 (self,event):
        wx.MessageBox("P10 is Open ","Pump", wx.OK|wx.ICON_INFORMATION)
```

```
#**********************                main definition and loop              ********************
#*****************************************************************************************************

def main():
    """Testing"""
    MyBackgroundPanel = wx.PySimpleApp(wx.Panel)
    f = MyFrame()
    d=ValveListBox()
    d.Show()
    f.Center()
    f.Show()
    MyBackgroundPanel.MainLoop()

if __name__ == "__main__":

    main()
```

19.4 Annex 2b: phython automation code including full automation

D:\AECENAR\MEGBI\MEGBI-VPP\AUT\CDMasterThesisHayssamHindy\GUI\101015_14MEGBI-VPP_automation.py

```
#-------------------------------------------------------------------------------
# Name:       MEGBI monitoring software
# Purpose:
#
# Author:      abd el rahman, Samir, hayssam
#
# Created:     09/05/2013
# Copyright:   (c) aecenar 2013, 2015
# Licence:     <AECENAR>
#-------------------------------------------------------------------------------

##!/usr/bin/env python
## -*- coding: iso-8859-15 -*-
import sys

#from tkinter import *
import threading
#from PIL import Image, ImageTk
```

```
import random

#import tkinter
import time
#from pymouse import PyMouse

#import ctypes
from ctypes import *

import wx
#import thread




#*********************          panel frame          **********************
#*****************************************************************************************
#wx.SetDefaultPyEncoding("iso-8859-15")
BACKGROUND_IMAGENAME = "220415MEGPI-VPP_PI_DIAGRAM.PNG"
#BACKGROUND_IMAGENAME =\"C:\Users\Hayssam\Desktop\GUI 220415MEGPI-VPP_PI_DIAGRAM.PNG"
##"hintergrundbild.jpg"




class MyBackgroundPanel(wx.Panel):

    def __init__(self, parent):
        wx.Panel.__init__(self, parent)
        self.bmp = wx.Bitmap(BACKGROUND_IMAGENAME)
        self.SetSize(self.bmp.GetSize())
        self.Bind(wx.EVT_PAINT, self.on_paint)
```

```
    def on_paint(self, event = None):
        dc = wx.BufferedPaintDC(self, self.bmp)


class MyFrame(wx.Frame):

    def __init__(self, parent = None, title = "MEGBI Vaccine Pilot Plant (MEGBI-VPP) Overview Upstream & Downstream Process"):

        self.testUSB            = True
        self.dll                = None
        self.USBAdr0            = 0
        self.USBAdr1            = 1
        self.USBAdr2            = 2
        self.USBOpened          = False
        self.counterUSBBoards   = 3

        wx.Frame.__init__(self, parent, -1, title)

        panel = MyBackgroundPanel(self)

        LABELSTYLE = wx.BORDER_SUNKEN | wx.ST_NO_AUTORESIZE | wx.ALIGN_CENTER_HORIZONTAL


        menuFile = wx.Menu()
        menuFile.Append(1, "&About...")
        menuFile.AppendSeparator()
        menuFile.Append(2, "Exit")
        menuBar = wx.MenuBar()
        menuBar.Append(menuFile, "File")
```

```
        self.SetMenuBar(menuBar)
        self.CreateStatusBar()
        self.SetStatusText("Welcome to MEGPI Project!")
        self.Bind(wx.EVT_MENU, self.OnAbout, id=1)
        self.Bind(wx.EVT_MENU, self.OnQuit, id=2)









            #Valves

                                #V1
        self.button_V_1 = wx.Button(panel, -1, "V1", pos=(95,100),size=(20,20))
        self.Bind(wx.EVT_BUTTON, self.V1, self.button_V_1)

                    #V2
        self.button_V_2 = wx.Button(panel, -1, "V2", pos=(150,90),size=(20,20))
        self.Bind(wx.EVT_BUTTON, self.V2, self.button_V_2)
                    #V3
        self.button_V_3 = wx.Button(panel, -1, "V3", pos=(150,135),size=(20,20))
        self.Bind(wx.EVT_BUTTON, self.V3, self.button_V_3)



                    #V4
```

```
self.button_V_4 = wx.Button(panel, -1, "V4", pos=(150,265),size=(20,20))
self.Bind(wx.EVT_BUTTON, self.V4, self.button_V_4)

        #V5
self.button_V_5 = wx.Button(panel, -1, "V5", pos=(210,135),size=(20,20))
self.Bind(wx.EVT_BUTTON, self.V5, self.button_V_5)

        #V6
self.button_V_6 = wx.Button(panel, -1, "V6", pos=(215,185),size=(20,20))
self.Bind(wx.EVT_BUTTON, self.V6, self.button_V_6)

        #V7
self.button_V_7 = wx.Button(panel, -1, "V7", pos=(215,220),size=(20,20))
self.Bind(wx.EVT_BUTTON, self.V7, self.button_V_7)

        #V8
self.button_V_8 = wx.Button(panel, -1, "V8", pos=(282,180),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V8, self.button_V_8)

        #V9
self.button_V_9 = wx.Button(panel, -1, "V9", pos=(282,158),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V9, self.button_V_9)

        #V10
self.button_V_10 = wx.Button(panel, -1, "V10", pos=(322,110),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V10, self.button_V_10)

        #V11
self.button_V_11 = wx.Button(panel, -1, "V11", pos=(380,250),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V11, self.button_V_11)

        #V12
self.button_V_12 = wx.Button(panel, -1, "V12", pos=(413,195),size=(25,20))
```

```
self.Bind(wx.EVT_BUTTON, self.V12, self.button_V_12)

        #V13
self.button_V_13 = wx.Button(panel, -1, "V13", pos=(380,130),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V13, self.button_V_13)

        #V14
self.button_V_14 = wx.Button(panel, -1, "V14", pos=(410,100),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V14, self.button_V_14)

        #V15
self.button_V_15 = wx.Button(panel, -1, "V15", pos=(460,112),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V15, self.button_V_15)

        #V16
self.button_V_16 = wx.Button(panel, -1, "V16", pos=(460,73),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V16, self.button_V_16)

        #V17
self.button_V_17 = wx.Button(panel, -1, "V17", pos=(460,38),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V17, self.button_V_17)



        #V18
self.button_V_18 = wx.Button(panel, -1, "V18", pos=(522,18),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V18, self.button_V_18)

        #V19
self.button_V_19 = wx.Button(panel, -1, "V19", pos=(635,18),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V19, self.button_V_19)
```

```
        #V20
self.button_V_20 = wx.Button(panel, -1, "V20", pos=(635,72),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V20, self.button_V_20)

        #V21
self.button_V_21 = wx.Button(panel, -1, "V21", pos=(675,112),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V21, self.button_V_21)

        #V22
self.button_V_22 = wx.Button(panel, -1, "V22", pos=(635,155),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V22, self.button_V_22)

        #V23
self.button_V_23 = wx.Button(panel, -1, "V23", pos=(780,18),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V23, self.button_V_23)

        #V24
self.button_V_24 = wx.Button(panel, -1, "V24", pos=(770,120),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V24, self.button_V_24)

        #V25
self.button_V_25 = wx.Button(panel, -1, "V25", pos=(770,155),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V25, self.button_V_25)

        #V26
self.button_V_26 = wx.Button(panel, -1, "V26", pos=(870,155),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V26, self.button_V_26)


        #V27
self.button_V_27 = wx.Button(panel, -1, "V27", pos=(510,185),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V27, self.button_V_27)
```

```
        #V28
    self.button_V_28 = wx.Button(panel, -1, "V28", pos=(620,185),size=(25,20))
    self.Bind(wx.EVT_BUTTON, self.V28, self.button_V_28)

        #V29
    self.button_V_29 = wx.Button(panel, -1, "V29", pos=(680,185),size=(25,20))
    self.Bind(wx.EVT_BUTTON, self.V29, self.button_V_29)



        #V30
    self.button_V_30 = wx.Button(panel, -1, "V30", pos=(620,240),size=(25,20))
    self.Bind(wx.EVT_BUTTON, self.V30, self.button_V_30)

        #V31
    self.button_V_31 = wx.Button(panel, -1, "V31", pos=(710,235),size=(25,20))
    self.Bind(wx.EVT_BUTTON, self.V31, self.button_V_31)

        #V32
    self.button_V_32 = wx.Button(panel, -1, "V32", pos=(950,182),size=(25,20))
    self.Bind(wx.EVT_BUTTON, self.V32, self.button_V_32)

        #V33
    self.button_V_33 = wx.Button(panel, -1, "V33", pos=(977,182),size=(25,20))
    self.Bind(wx.EVT_BUTTON, self.V33, self.button_V_33)



        #V34
    self.button_V_34 = wx.Button(panel, -1, "V34", pos=(1083,188),size=(25,20))
    self.Bind(wx.EVT_BUTTON, self.V34, self.button_V_34)
```

```
        #V35
self.button_V_35 = wx.Button(panel, -1, "V35", pos=(1083,220),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V35, self.button_V_35)

        #V36
self.button_V_36 = wx.Button(panel, -1, "V36", pos=(1170,110),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V36, self.button_V_36)

        #V37
self.button_V_37 = wx.Button(panel, -1, "V37", pos=(1200,110),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V37, self.button_V_37)

        #V38
self.button_V_38 = wx.Button(panel, -1, "V38", pos=(1240,150),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V38, self.button_V_38)

        #V39
self.button_V_39 = wx.Button(panel, -1, "V39", pos=(1273,205),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V39, self.button_V_39)



        #V40
self.button_V_40 = wx.Button(panel, -1, "V40", pos=(483,314),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V40, self.button_V_40)

        #V41
self.button_V_41 = wx.Button(panel, -1, "V41", pos=(478,398),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V41, self.button_V_41)

        #V42
self.button_V_42 = wx.Button(panel, -1, "V42", pos=(540,314),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V42, self.button_V_42)
```

```
        #V43
self.button_V_43 = wx.Button(panel, -1, "V43", pos=(548,398),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V43, self.button_V_43)


        #V44
self.button_V_44 = wx.Button(panel, -1, "V44", pos=(580,300),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V44, self.button_V_44)

        #V45
self.button_V_45 = wx.Button(panel, -1, "V45", pos=(620,314),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V45, self.button_V_45)



        #V46
self.button_V_46 = wx.Button(panel, -1, "V46", pos=(760,440),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V46, self.button_V_46)

        #V47
self.button_V_47 = wx.Button(panel, -1, "V47", pos=(760,400),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V47, self.button_V_47)

        #V48
self.button_V_48 = wx.Button(panel, -1, "V48", pos=(783,375),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V48, self.button_V_48)

        #V49
self.button_V_49 = wx.Button(panel, -1, "V49", pos=(880,428),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V49, self.button_V_49)
```

```
        #V50
self.button_V_50 = wx.Button(panel, -1, "V50", pos=(970,377),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V50, self.button_V_50)

        #V51
self.button_V_51 = wx.Button(panel, -1, "V51", pos=(963,450),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V51, self.button_V_51)

        #V52
self.button_V_52 = wx.Button(panel, -1, "V52", pos=(1005,470),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V52, self.button_V_52)

        #V53
self.button_V_53 = wx.Button(panel, -1, "V53", pos=(1030,395),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V53, self.button_V_53)

        #V54
self.button_V_54 = wx.Button(panel, -1, "V54", pos=(1080,395),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V54, self.button_V_54)
        #V55
self.button_V_55 = wx.Button(panel, -1, "V55", pos=(1118,377),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V55, self.button_V_55)

        #V56
self.button_V_56 = wx.Button(panel, -1, "V56", pos=(1158,468),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V56, self.button_V_56)

        #V57
self.button_V_57 = wx.Button(panel, -1, "V57", pos=(1190,420),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.V57, self.button_V_57)
```

```
            #V58
        self.button_V_58 = wx.Button(panel, -1, "V58", pos=(710,580),size=(25,20))
        self.Bind(wx.EVT_BUTTON, self.V58, self.button_V_58)



        #Pumps

            #P1
        self.button_P_1 = wx.Button(panel, -1, "P1", pos=(150,180),size=(20,20))
        self.Bind(wx.EVT_BUTTON, self.P1, self.button_P_1)

            #P2
        self.button_P_2 = wx.Button(panel, -1, "P2", pos=(150,220),size=(20,20))
        self.Bind(wx.EVT_BUTTON, self.P2, self.button_P_2)

            #P3
        self.button_P_3 = wx.Button(panel, -1, "P3", pos=(510,40),size=(20,20))
        self.Bind(wx.EVT_BUTTON, self.P3, self.button_P_3)

            #P4
        self.button_P_4 = wx.Button(panel, -1, "P4", pos=(880,18),size=(20,20))
        self.Bind(wx.EVT_BUTTON, self.P4, self.button_P_4)

            #P5
        self.button_P_5 = wx.Button(panel, -1, "P5", pos=(730,187),size=(20,20))
        self.Bind(wx.EVT_BUTTON, self.P5, self.button_P_5)

            #P6
        self.button_P_6 = wx.Button(panel, -1, "P6", pos=(1083,130),size=(20,20))
        self.Bind(wx.EVT_BUTTON, self.P6, self.button_P_6)
```

```
            #P7
self.button_P_7 = wx.Button(panel, -1, "P7", pos=(380,328),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.P7, self.button_P_7)

            #P8
self.button_P_8 = wx.Button(panel, -1, "P8", pos=(710,423),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.P8, self.button_P_8)

            #P9
self.button_P_9 = wx.Button(panel, -1, "P9", pos=(900,380),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.P9, self.button_P_9)

            #P10
self.button_P_10 = wx.Button(panel, -1, "P10", pos=(1200,380),size=(25,20))
self.Bind(wx.EVT_BUTTON, self.P10, self.button_P_10)

        #control buttons

                        #Turn ON button
self.button_Turn_ON = wx.Button(panel, -1, "Turn On", pos=(40,40),size=(60,20))
self.Bind(wx.EVT_BUTTON, self.MEGBIVPPFullAutomationStart, self.button_Turn_ON)
self.ON = wx.StaticText(panel, size = (40, -1), pos = (140, 40), style = LABELSTYLE)




   #Text column 1
wx.StaticText(panel,20," Valve Selected : ",(10,270))
wx.StaticText(panel,-1," V1 ",(18,300))
wx.StaticText(panel,-1," V2 ",(18,326))
wx.StaticText(panel,-1," V3 ",(18,352))
wx.StaticText(panel,-1," V4 ",(18,378))
```

```
wx.StaticText(panel,-1," V5 ",(18,402))
wx.StaticText(panel,-1," V6 ",(18,428))
wx.StaticText(panel,-1," V7 ",(18,454))
wx.StaticText(panel,-1," V8 ",(18,480))
wx.StaticText(panel,-1," V9 ",(18,506))
wx.StaticText(panel,-1," V10 ",(18,532))
wx.StaticText(panel,-1," V11 ",(18,558))
wx.StaticText(panel,-1," V12 ",(18,584))

   #Text column 2
wx.StaticText(panel,-1," V13 ",(85,300))
wx.StaticText(panel,-1," V14 ",(85,326))
wx.StaticText(panel,-1," V15 ",(85,352))
wx.StaticText(panel,-1," V16 ",(85,378))
wx.StaticText(panel,-1," V17 ",(85,402))
wx.StaticText(panel,-1," V18 ",(85,428))
wx.StaticText(panel,-1," V19 ",(85,454))
wx.StaticText(panel,-1," V20 ",(85,480))
wx.StaticText(panel,-1," V21 ",(85,506))
wx.StaticText(panel,-1," V22 ",(85,532))
wx.StaticText(panel,-1," V23 ",(85,558))
wx.StaticText(panel,-1," V24 ",(85,584))

 #Text column 3
wx.StaticText(panel,-1," V25 ",(155,300))
wx.StaticText(panel,-1," V26 ",(155,326))
wx.StaticText(panel,-1," V27 ",(155,352))
wx.StaticText(panel,-1," V28 ",(155,378))
wx.StaticText(panel,-1," V29 ",(155,402))
wx.StaticText(panel,-1," V30 ",(155,428))
wx.StaticText(panel,-1," V31 ",(155,454))
wx.StaticText(panel,-1," V32 ",(155,480))
```

```
wx.StaticText(panel,-1," V33 ",(155,506))
wx.StaticText(panel,-1," V34 ",(155,532))
wx.StaticText(panel,-1," V35 ",(155,558))
wx.StaticText(panel,-1," V36 ",(155,584))

#Text column 4
wx.StaticText(panel,-1," V37 ",(223,300))
wx.StaticText(panel,-1," V38 ",(223,326))
wx.StaticText(panel,-1," V39 ",(223,352))
wx.StaticText(panel,-1," V40 ",(223,378))
wx.StaticText(panel,-1," V41 ",(223,402))
wx.StaticText(panel,-1," V42 ",(223,428))
wx.StaticText(panel,-1," V43 ",(223,454))
wx.StaticText(panel,-1," V44 ",(223,480))
wx.StaticText(panel,-1," V45 ",(223,506))
wx.StaticText(panel,-1," V46 ",(223,532))
wx.StaticText(panel,-1," V47 ",(223,558))
wx.StaticText(panel,-1," V48 ",(223,584))

#Text column 5
wx.StaticText(panel,-1," V49 ",(293,300))
wx.StaticText(panel,-1," V50 ",(293,326))
wx.StaticText(panel,-1," V51 ",(293,352))
wx.StaticText(panel,-1," V52 ",(293,378))
wx.StaticText(panel,-1," V53 ",(293,402))
wx.StaticText(panel,-1," V54 ",(293,428))
wx.StaticText(panel,-1," V55 ",(293,454))
wx.StaticText(panel,-1," V56 ",(293,480))
wx.StaticText(panel,-1," V57 ",(293,506))
wx.StaticText(panel,-1," V58 ",(293,532))
#wx.StaticText(panel,-1," V59 ",(293,558))
# wx.StaticText(panel,-1," V60 ",(293,584))
```

```
    #Label  Column 1
self.Valve1 = wx.StaticText(panel, size = (26, -1), pos = (45, 300), style = LABELSTYLE)
self.Valve2 = wx.StaticText(panel, size = (26, -1), pos = (45, 326), style = LABELSTYLE)
self.Valve3 = wx.StaticText(panel, size = (26, -1), pos = (45, 352), style = LABELSTYLE)
self.Valve4 = wx.StaticText(panel, size = (26, -1), pos = (45, 378), style = LABELSTYLE)
self.Valve5 = wx.StaticText(panel, size = (26, -1), pos = (45, 402), style = LABELSTYLE)
self.Valve6 = wx.StaticText(panel, size = (26, -1), pos = (45, 428), style = LABELSTYLE)
self.Valve7 = wx.StaticText(panel, size = (26, -1), pos = (45, 454), style = LABELSTYLE)
self.Valve8 = wx.StaticText(panel, size = (26, -1), pos = (45, 480), style = LABELSTYLE)
self.Valve9 = wx.StaticText(panel, size = (26, -1), pos = (45, 506), style = LABELSTYLE)
self.Valve10 = wx.StaticText(panel, size = (26, -1), pos = (45, 532), style = LABELSTYLE)
self.Valve11 = wx.StaticText(panel, size = (26, -1), pos = (45, 558), style = LABELSTYLE)
self.Valve12 = wx.StaticText(panel, size = (26, -1), pos = (45, 584), style = LABELSTYLE)


    #Label column 2
self.Valve13 = wx.StaticText(panel, size = (26, -1), pos = (115, 300), style = LABELSTYLE)
self.Valve14 = wx.StaticText(panel, size = (26, -1), pos = (115, 326), style = LABELSTYLE)
self.Valve15 = wx.StaticText(panel, size = (26, -1), pos = (115, 352), style = LABELSTYLE)
self.Valve16 = wx.StaticText(panel, size = (26, -1), pos = (115, 378), style = LABELSTYLE)
self.Valve17 = wx.StaticText(panel, size = (26, -1), pos = (115, 402), style = LABELSTYLE)
```

```
self.Valve18 = wx.StaticText(panel, size = (26, -1), pos = (115, 428), style = LABELSTYLE)
self.Valve19 = wx.StaticText(panel, size = (26, -1), pos = (115, 454), style = LABELSTYLE)
self.Valve20 = wx.StaticText(panel, size = (26, -1), pos = (115, 480), style = LABELSTYLE)
self.Valve21 = wx.StaticText(panel, size = (26, -1), pos = (115, 506), style = LABELSTYLE)
self.Valve22 = wx.StaticText(panel, size = (26, -1), pos = (115, 532), style = LABELSTYLE)
self.Valve23 = wx.StaticText(panel, size = (26, -1), pos = (115, 558), style = LABELSTYLE)
self.Valve24 = wx.StaticText(panel, size = (26, -1), pos = (115, 584), style = LABELSTYLE)

 #Label column 3

self.Valve25 = wx.StaticText(panel, size = (26, -1), pos = (185, 300), style = LABELSTYLE)
self.Valve26 = wx.StaticText(panel, size = (26, -1), pos = (185, 326), style = LABELSTYLE)
self.Valve27 = wx.StaticText(panel, size = (26, -1), pos = (185, 352), style = LABELSTYLE)
self.Valve28 = wx.StaticText(panel, size = (26, -1), pos = (185, 378), style = LABELSTYLE)
self.Valve29 = wx.StaticText(panel, size = (26, -1), pos = (185, 402), style = LABELSTYLE)
self.Valve30 = wx.StaticText(panel, size = (26, -1), pos = (185, 428), style = LABELSTYLE)
self.Valve31 = wx.StaticText(panel, size = (26, -1), pos = (185, 454), style = LABELSTYLE)
self.Valve32 = wx.StaticText(panel, size = (26, -1), pos = (185, 480), style = LABELSTYLE)
self.Valve33 = wx.StaticText(panel, size = (26, -1), pos = (185, 506), style = LABELSTYLE)
self.Valve34 = wx.StaticText(panel, size = (26, -1), pos = (185, 532), style = LABELSTYLE)
self.Valve35 = wx.StaticText(panel, size = (26, -1), pos = (185, 558), style = LABELSTYLE)
self.Valve36 = wx.StaticText(panel, size = (26, -1), pos = (185, 584), style = LABELSTYLE)

 #Label column 4

self.Valve37 = wx.StaticText(panel, size = (26, -1), pos = (253, 300), style = LABELSTYLE)
self.Valve38 = wx.StaticText(panel, size = (26, -1), pos = (253, 326), style = LABELSTYLE)
self.Valve39 = wx.StaticText(panel, size = (26, -1), pos = (253, 352), style = LABELSTYLE)
self.Valve40 = wx.StaticText(panel, size = (26, -1), pos = (253, 378), style = LABELSTYLE)
self.Valve41 = wx.StaticText(panel, size = (26, -1), pos = (253, 402), style = LABELSTYLE)
self.Valve42 = wx.StaticText(panel, size = (26, -1), pos = (253, 428), style = LABELSTYLE)
self.Valve43 = wx.StaticText(panel, size = (26, -1), pos = (253, 454), style = LABELSTYLE)
self.Valve44 = wx.StaticText(panel, size = (26, -1), pos = (253, 480), style = LABELSTYLE)
```

```
        self.Valve45 = wx.StaticText(panel, size = (26, -1), pos = (253, 506), style = LABELSTYLE)
        self.Valve46 = wx.StaticText(panel, size = (26, -1), pos = (253, 532), style = LABELSTYLE)
        self.Valve47 = wx.StaticText(panel, size = (26, -1), pos = (253, 558), style = LABELSTYLE)
        self.Valve48 = wx.StaticText(panel, size = (26, -1), pos = (253, 584), style = LABELSTYLE)


        #Label column 5


        self.Valve49 = wx.StaticText(panel, size = (26, -1), pos = (323, 300), style = LABELSTYLE)
        self.Valve50 = wx.StaticText(panel, size = (26, -1), pos = (323, 326), style = LABELSTYLE)
        self.Valve51 = wx.StaticText(panel, size = (26, -1), pos = (323, 352), style = LABELSTYLE)
        self.Valve52 = wx.StaticText(panel, size = (26, -1), pos = (323, 378), style = LABELSTYLE)
        self.Valve53 = wx.StaticText(panel, size = (26, -1), pos = (323, 402), style = LABELSTYLE)
        self.Valve54 = wx.StaticText(panel, size = (26, -1), pos = (323, 428), style = LABELSTYLE)
        self.Valve55 = wx.StaticText(panel, size = (26, -1), pos = (323, 454), style = LABELSTYLE)
        self.Valve56 = wx.StaticText(panel, size = (26, -1), pos = (323, 480), style = LABELSTYLE)
        self.Valve57 = wx.StaticText(panel, size = (26, -1), pos = (323, 506), style = LABELSTYLE)
        self.Valve58 = wx.StaticText(panel, size = (26, -1), pos = (323, 532), style = LABELSTYLE)
        #self.Valve59 = wx.StaticText(panel, size = (26, -1), pos = (323, 558), style = LABELSTYLE)

######temperature sensors
        wx.StaticText(panel,-1," Temperature : ",(1100,592))
        self.temp = wx.StaticText(panel, size = (40, -1), pos = (1200, 590), style = LABELSTYLE)
```

```
#*********************          Run USB System          **********************
#*****************************************************************************************
   def OpenUSBBoardThread(self):
      self.dll = windll.K8061
      i = self.counterUSBBoards
      for doit in range(0,i+1):
         try:
            self.dll.OpenDevice()
            self.USBOpened = True
# debug info
            print ('USB Board is now connected!')
#end debug info
         except:
            txt = ('Please Check USB Board connection')
            print ('txt')
            return




#####################################################


   def OnAbout(self, event):
      wx.MessageBox("This is a Screen controller of MEGPI Project","Welcome to my python", wx.OK | wx.ICON_INFORMATION, self)
   def OnQuit(self, event):
      self.Close()
   def on_timer(self, event = None):
      self.Close()


   def Sensors(self,event):
      answer = self.dll.ReadAnalogChannel(0,1)
      new_value = str(answer)
```

```
        self.temp.SetLabel(answer)
        #power for the temperature sensor (255 >>>> 5V power)
        self.dll.OutputAnalogChannel(0,6,255)

#*********************          Define Valvue          ********************
#*************************************************************************************************
    def MEGBIVPPFullAutomationStart (self, event):

        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'



        if self.ON.GetLabel()==valve_status_OFF:
            self.ON.SetLabel(valve_status_ON)
            self.OpenUSBBoardThread()
            self.timer = wx.Timer()
            self.timer.Bind(wx.EVT_TIMER, self.on_timer)
            self.timer.Start(100)
            print'system is turning on'
            self.dll.SetDigitalChannel(0,1)
            print'V1 is open'
            self.Valve1.SetLabel(valve_status_ON)
            time.sleep(5)
            self.dll.SetDigitalChannel(0,2)
            print'V2 is open'
            self.Valve2.SetLabel(valve_status_ON)
            self.timer.Start(40)
            time.sleep(20)
            self.dll.ClearDigitalChannel(0,1)
            self.dll.ClearDigitalChannel(0,2)
            print'V1 is close'
```

```
self.Valve1.SetLabel(valve_status_OFF)
print'V2 is close'
self.Valve2.SetLabel(valve_status_OFF)
self.dll.SetDigitalChannel(0,3)
print'V3 is open'
self.Valve3.SetLabel(valve_status_ON)
self.timer.Start(40)
time.sleep(20)
self.dll.SetDigitalChannel(0,4)
self.dll.ClearDigitalChannel(0,3)
self.Valve3.SetLabel(valve_status_OFF)
print'V3 is close'
print'V4 is open'
self.Valve4.SetLabel(valve_status_ON)
#self.timer.Start(30)
#time.sleep(20)
self.dll.SetDigitalChannel(0,5)
self.dll.ClearDigitalChannel(0,4)
print'V4 i close'

self.Valve4.SetLabel(valve_status_OFF)
print'V5 is open'
self.Valve5.SetLabel(valve_status_ON)
self.dll.SetDigitalChannel(0,6)
print'P1 is turn on'
#self.timer.Start(400)
time.sleep(20)
self.dll.ClearDigitalChannel(0,5)
self.dll.ClearDigitalChannel(0,6)
print'V5 is close'
self.Valve5.SetLabel(valve_status_OFF)
print'P1 is turn off'
self.dll.SetDigitalChannel(0,7)
```

```
print'V6 is open'
self.Valve6.SetLabel(valve_status_ON)
#self.timer.Start(40)
time.sleep(5)
self.dll.ClearDigitalChannel(0,7)
print'V6 is close'
self.Valve6.SetLabel(valve_status_OFF)
self.dll.SetDigitalChannel(0,8)
print'V7 is open'
self.Valve7.SetLabel(valve_status_ON)
#self.timer.Start(400)
time.sleep(20)
self.dll.OutputAnalogChannel(0,2,255)
print'V8 is open'
self.Valve8.SetLabel(valve_status_ON)
self.dll.ClearDigitalChannel(0,8)
print'V7 is close'
self.Valve7.SetLabel(valve_status_OFF)
#self.timer.Start(40)
time.sleep(5)
self.dll.OutputAnalogChannel(0,2,0)
print'V8 is close'
self.Valve8.SetLabel(valve_status_OFF)
self.dll.OutputAnalogChannel(0,3,255)
print'V9 is open'
self.Valve9.SetLabel(valve_status_ON)
self.dll.OutputAnalogChannel(0,4,255)
print'P2 is turn on'
#self.timer.Start(400)
time.sleep(20)
self.dll.OutputAnalogChannel(0,3,0)
self.dll.OutputAnalogChannel(0,4,0)
```

```
self.dll.OutputAnalogChannel(0,5,255)
print'V9 is close'
self.Valve9.SetLabel(valve_status_OFF)
print'P2 is turn off'
print'V10 is open'
self.Valve10.SetLabel(valve_status_ON)
#self.timer.Start(40)
time.sleep(5)
self.dll.OutputAnalogChannel(0,5,0)
print'V10 is close'
self.Valve10.SetLabel(valve_status_OFF)
self.dll.OutputAnalogChannel(0,1,255)
print'V11 is open'
self.Valve11.SetLabel(valve_status_ON)
self.timer.Start(400)
time.sleep(20)
self.dll.OutputAnalogChannel(0,1,0)
self.dll.OutputAnalogChannel(0,7,255)
print'V11 is close'
self.Valve11.SetLabel(valve_status_OFF)
print'V12 is open'
self.Valve12.SetLabel(valve_status_ON)
#self.timer.Start(40)
time.sleep(5)
self.dll.OutputAnalogChannel(0,7,0)
print'V12 is close'
self.Valve12.SetLabel(valve_status_OFF)
self.dll.OutputAnalogChannel(0,8,255)
print'V13 is open'
self.Valve13.SetLabel(valve_status_ON)
#self.timer.Start(400)
time.sleep(20)
self.dll.OutputAnalogChannel(0,8,0)
```

```
self.dll.SetDigitalChannel(1,1)
print'V13 is close'
self.Valve13.SetLabel(valve_status_OFF)
print'V14 is open'
self.Valve14.SetLabel(valve_status_ON)
self.dll.SetDigitalChannel(1,2)
print'P3 is turn on'
#self.timer.Start(40)
time.sleep(5)
self.dll.ClearDigitalChannel(1,1)
self.dll.ClearDigitalChannel(1,2)
print'V14 is close'
self.Valve14.SetLabel(valve_status_OFF)
print'P3 is turn on'
self.dll.SetDigitalChannel(1,3)
print'V15 is open'
self.Valve15.SetLabel(valve_status_ON)
#self.timer.Start(50)
time.sleep(5)
self.dll.ClearDigitalChannel(1,3)
print'V15 is close'
self.Valve15.SetLabel(valve_status_OFF)
self.timer.Start(100)
self.dll.SetDigitalChannel(1,4)
print'V16 is open'
self.Valve16.SetLabel(valve_status_ON)
#self.timer.Start(20)
time.sleep(10)
self.dll.ClearDigitalChannel(1,4)
self.dll.SetDigitalChannel(1,5)
self.dll.SetDigitalChannel(1,6)
print'V16 is close'
```

```
self.Valve16.SetLabel(valve_status_OFF)
print'V17 is open'
self.Valve17.SetLabel(valve_status_ON)
print'V18 is open'
self.Valve18.SetLabel(valve_status_ON)
#self.timer.Start(500)
time.sleep(20)
self.dll.ClearDigitalChannel(1,5)
self.dll.ClearDigitalChannel(1,6)
print'V17 is close'
self.Valve17.SetLabel(valve_status_OFF)
print'V18 is close'
self.Valve18.SetLabel(valve_status_OFF)
self.dll.SetDigitalChannel(1,7)
print'V19 is open'
self.Valve19.SetLabel(valve_status_ON)
self.dll.SetDigitalChannel(1,8)
print'P4 is turn on'
#self.timer.Start(50)
time.sleep(10)
self.dll.ClearDigitalChannel(1,7)
print'V19 is close'
self.Valve19.SetLabel(valve_status_OFF)
self.dll.ClearDigitalChannel(1,8)
print'P4 is turn off'
self.dll.OutputAnalogChannel(1,1,255)
print'V20 is open'
self.Valve20.SetLabel(valve_status_ON)
#self.timer.Start(50)
time.sleep(5)
self.dll.OutputAnalogChannel(1,1,0)
print'V20 is close'
self.Valve20.SetLabel(valve_status_OFF)
```

```
self.dll.OutputAnalogChannel(1,2,255)
self.dll.OutputAnalogChannel(1,3,255)
print'V21 is open'
self.Valve21.SetLabel(valve_status_ON)
print'V22 is open'
self.Valve22.SetLabel(valve_status_ON)
#self.timer.Start(500)
time.sleep(20)
self.dll.OutputAnalogChannel(1,2,0)
self.dll.OutputAnalogChannel(1,3,0)
print'V21 is close'
self.Valve21.SetLabel(valve_status_OFF)
print'V22 is close'
self.Valve22.SetLabel(valve_status_OFF)
self.dll.OutputAnalogChannel(1,4,255)
self.dll.OutputAnalogChannel(1,5,255)
print'V23 is open'
self.Valve23.SetLabel(valve_status_ON)
print'P5 is turn On'
#self.timer.Start(40)
time.sleep(5)
self.dll.OutputAnalogChannel(1,4,0)
self.dll.OutputAnalogChannel(1,5,0)
print'V23 is close'
self.Valve23.SetLabel(valve_status_OFF)
print'P5 is turn off'
self.dll.OutputAnalogChannel(1,6,255)
print'V24 is open'
self.Valve24.SetLabel(valve_status_ON)
#self.timer.Start(500)
time.sleep(20)
self.dll.OutputAnalogChannel(1,6,0)
```

```
print'V24 is close'
self.Valve24.SetLabel(valve_status_OFF)
self.dll.OutputAnalogChannel(1,7,255)
print'V25 is open'
self.Valve25.SetLabel(valve_status_ON)
#self.timer.Start(50)
time.sleep(5)
self.dll.OutputAnalogChannel(1,7,0)
print'V25 is close'
self.Valve25.SetLabel(valve_status_OFF)
self.dll.OutputAnalogChannel(1,8,255)
print'V26 is open'
self.Valve26.SetLabel(valve_status_ON)
#self.timer.Start(600)
time.sleep(20)
self.dll.OutputAnalogChannel(1,8,0)
print'V26 is close'
self.Valve26.SetLabel(valve_status_OFF)
self.dll.SetDigitalChannel(2,1)
print'V27 is open'
self.Valve27.SetLabel(valve_status_ON)
#self.timer.Start(40)
time.sleep(5)
self.dll.ClearDigitalChannel(2,1)
print'V27 is close'
self.Valve27.SetLabel(valve_status_OFF)
self.dll.SetDigitalChannel(2,2)
print'V28 is open'
self.Valve28.SetLabel(valve_status_ON)
#self.timer.Start(60)
time.sleep(10)
self.dll.SetDigitalChannel(2,3)
self.dll.SetDigitalChannel(2,4)
```

```
print'V29 is open'
self.Valve29.SetLabel(valve_status_ON)
print'V30 is open'
self.Valve30.SetLabel(valve_status_ON)
self.dll.ClearDigitalChannel(2,2)
print'V28 is close'
self.Valve28.SetLabel(valve_status_OFF)
#self.timer.Start(40)
time.sleep(5)
self.dll.ClearDigitalChannel(2,3)
self.dll.ClearDigitalChannel(2,4)
print'V29 is close'
self.Valve29.SetLabel(valve_status_OFF)
print'V30 is close'
self.Valve30.SetLabel(valve_status_OFF)
#self.timer.Start(600)
time.sleep(20)
self.dll.SetDigitalChannel(2,5)
print'V31 is open'
self.Valve31.SetLabel(valve_status_ON)
self.dll.SetDigitalChannel(2,6)
print'P6 is turn on'
#self.timer.Start(50)
time.sleep(5)
self.dll.ClearDigitalChannel(2,5)
self.dll.ClearDigitalChannel(2,6)
print'V31 is close'
self.Valve31.SetLabel(valve_status_OFF)
print'P6 is turn off'
#self.timer.Start(40)
time.sleep(5)
self.dll.SetDigitalChannel(2,7)
```

```
print'V32 is open'
self.Valve32.SetLabel(valve_status_ON)
#self.timer.Start(600)
time.sleep(20)
print'V32 is close'
self.Valve32.SetLabel(valve_status_OFF)
self.dll.ClearDigitalChannel(2,7)
self.dll.SetDigitalChannel(2,8)
print'V33 is open'
self.Valve33.SetLabel(valve_status_ON)
#self.timer.Start(50)
time.sleep(5)
self.dll.ClearDigitalChannel(2,8)
print'V33 is close'
self.Valve33.SetLabel(valve_status_OFF)
self.dll.OutputAnalogChannel(2,1,255)
print'V34 is open'
self.Valve34.SetLabel(valve_status_ON)
#self.timer.Start(50)
time.sleep(5)
self.dll.OutputAnalogChannel(2,1,0)
print'V34 is close'
self.Valve34.SetLabel(valve_status_OFF)
self.dll.OutputAnalogChannel(2,2,255)

self.dll.OutputAnalogChannel(2,3,255)
print'V35 is open'
self.Valve35.SetLabel(valve_status_ON)
print'P7 is turn on'
#self.timer.Start(50)
time.sleep(5)
self.dll.OutputAnalogChannel(2,2,0)
self.dll.OutputAnalogChannel(2,3,0)
```

```
        print'V35 is close'
        print'P7 is turn off'
        print'System is turning off'



    else:
        self.ON.SetLabel(valve_status_OFF)
        wx.MessageBox("Turning OFF ","System", wx.OK|wx.ICON_INFORMATION)









        #self.dll.OutputAnalogChannel(3,8,255)
        #
        #def V1(self,event):
         #   valve_status_ON = 'ON'
          #  valve_status_OFF = 'OFF'


           # self.OpenUSBBoardThread()
            #time.sleep(0.5)
            #self.dll.SetDigitalChannel(0,1)
            #self.timer = wx.Timer()
            #print'V1 is open'
            #self.V1.Open()
            #self.timer.Bind(wx.EVT_TIMER, self.on_timer)
            #self.timer.Start(120)
        #def V2 (self,event):
         #   self.OpenUSBBoardThread()
          #  self.dll.SetDigitalChannel(0,2)
```

```
            # self.Valve1.SetLabel(valve_status_ON)


        #else:
            #self.ON.SetLabel(valve_status_ON)
            #wx.MessageBox("Turning ON ","System", wx.OK|wx.ICON_INFORMATION)



    def V1(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'

            if self.Valve1.GetLabel()==valve_status_ON:
                self.Valve1.SetLabel(valve_status_OFF)
               # wx.MessageBox("V1 is Open ","Open", wx.OK|wx.ICON_INFORMATION)
            else:
                self.Valve1.SetLabel(valve_status_ON)
                #wx.MessageBox("V1 is Close","Closed", wx.OK|wx.ICON_INFORMATION)




    def V2(self, event):
            valve_status_ON = 'ON'
            valve_status_OFF = 'OFF'

            if self.Valve2.GetLabel()==valve_status_ON:
                self.Valve2.SetLabel(valve_status_OFF)
               # wx.MessageBox("V1 is Open ","Open", wx.OK|wx.ICON_INFORMATION)
            else:
                self.Valve2.SetLabel(valve_status_ON)
                #wx.MessageBox("V1 is Close","Closed", wx.OK|wx.ICON_INFORMATION)
```

```
    def V3(self, event):


        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'


        if self.Valve3.GetLabel()==valve_status_ON:
            self.Valve3.SetLabel(valve_status_OFF)
           # wx.MessageBox("V1 is Open ","Open", wx.OK|wx.ICON_INFORMATION)
        else:
            self.Valve3.SetLabel(valve_status_ON)
            #wx.MessageBox("V1 is Close","Closed", wx.OK|wx.ICON_INFORMATION)


    def V4(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'


        if self.Valve4.GetLabel()==valve_status_ON:
            self.Valve4.SetLabel(valve_status_OFF)
           # wx.MessageBox("V1 is Open ","Open", wx.OK|wx.ICON_INFORMATION)
        else:
           self.Valve4.SetLabel(valve_status_ON)
            #wx.MessageBox("V1 is Close","Closed", wx.OK|wx.ICON_INFORMATION)


    def V5(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'


        if self.Valve5.GetLabel()==valve_status_ON:
            self.Valve5.SetLabel(valve_status_OFF)
           # wx.MessageBox("V1 is Open ","Open", wx.OK|wx.ICON_INFORMATION)
        else:
            self.Valve5.SetLabel(valve_status_ON)
```

```
            #wx.MessageBox("V1 is Close","Closed", wx.OK|wx.ICON_INFORMATION)

    def V6(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve6.GetLabel()==valve_status_ON:
            self.Valve6.SetLabel(valve_status_OFF)
           # wx.MessageBox("V1 is Open ","Open", wx.OK|wx.ICON_INFORMATION)
        else:
            self.Valve6.SetLabel(valve_status_ON)
            #wx.MessageBox("V1 is Close","Closed", wx.OK|wx.ICON_INFORMATION)

    def V7(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve7.GetLabel()==valve_status_ON:
            self.Valve7.SetLabel(valve_status_OFF)
           # wx.MessageBox("V1 is Open ","Open", wx.OK|wx.ICON_INFORMATION)
        else:
            self.Valve7.SetLabel(valve_status_ON)
            #wx.MessageBox("V1 is Close","Closed", wx.OK|wx.ICON_INFORMATION)

    def V8(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve8.GetLabel()==valve_status_ON:
            self.Valve8.SetLabel(valve_status_OFF)
           # wx.MessageBox("V1 is Open ","Open", wx.OK|wx.ICON_INFORMATION)
        else:
            self.Valve8.SetLabel(valve_status_ON)
```

```
            #wx.MessageBox("V1 is Close","Closed", wx.OK|wx.ICON_INFORMATION)


    def V9(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve9.GetLabel()==valve_status_ON:
            self.Valve9.SetLabel(valve_status_OFF)
           # wx.MessageBox("V1 is Open ","Open", wx.OK|wx.ICON_INFORMATION)
        else:
            self.Valve9.SetLabel(valve_status_ON)
            #wx.MessageBox("V1 is Close","Closed", wx.OK|wx.ICON_INFORMATION)


    def V10(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve10.GetLabel()==valve_status_ON:
            self.Valve10.SetLabel(valve_status_OFF)


        else:
            self.Valve10.SetLabel(valve_status_ON)



    def V11(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve11.GetLabel()==valve_status_ON:
            self.Valve11.SetLabel(valve_status_OFF)


        else:
```

```
            self.Valve11.SetLabel(valve_status_ON)

    def V12(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve12.GetLabel()==valve_status_ON:
            self.Valve12.SetLabel(valve_status_OFF)

        else:
            self.Valve12.SetLabel(valve_status_ON)

    def V13(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve13.GetLabel()==valve_status_ON:
            self.Valve13.SetLabel(valve_status_OFF)

        else:
            self.Valve13.SetLabel(valve_status_ON)

    def V14(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve14.GetLabel()==valve_status_ON:
            self.Valve14.SetLabel(valve_status_OFF)

        else:
            self.Valve14.SetLabel(valve_status_ON)

    def V15(self, event):
```

```
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve15.GetLabel()==valve_status_ON:
            self.Valve15.SetLabel(valve_status_OFF)

        else:
            self.Valve15.SetLabel(valve_status_ON)

    def V16(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve16.GetLabel()==valve_status_ON:
            self.Valve16.SetLabel(valve_status_OFF)

        else:
            self.Valve16.SetLabel(valve_status_ON)
    def V17(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve17.GetLabel()==valve_status_ON:
            self.Valve17.SetLabel(valve_status_OFF)

        else:
            self.Valve17.SetLabel(valve_status_ON)

    def V18(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'
```

```
        if self.Valve18.GetLabel()==valve_status_ON:
            self.Valve18.SetLabel(valve_status_OFF)

        else:
            self.Valve18.SetLabel(valve_status_ON)

    def V19(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve19.GetLabel()==valve_status_ON:
            self.Valve19.SetLabel(valve_status_OFF)

        else:
            self.Valve19.SetLabel(valve_status_ON)

    def V20(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve20.GetLabel()==valve_status_ON:
            self.Valve20.SetLabel(valve_status_OFF)

        else:
            self.Valve20.SetLabel(valve_status_ON)

    def V21(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve21.GetLabel()==valve_status_ON:
            self.Valve21.SetLabel(valve_status_OFF)
```

```
        else:
            self.Valve21.SetLabel(valve_status_ON)

    def V22(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve22.GetLabel()==valve_status_ON:
            self.Valve22.SetLabel(valve_status_OFF)

        else:
            self.Valve22.SetLabel(valve_status_ON)

    def V23(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve23.GetLabel()==valve_status_ON:
            self.Valve23.SetLabel(valve_status_OFF)

        else:
            self.Valve23.SetLabel(valve_status_ON)

    def V24(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve24.GetLabel()==valve_status_ON:
            self.Valve24.SetLabel(valve_status_OFF)

        else:
            self.Valve24.SetLabel(valve_status_ON)
```

```
def V25(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve25.GetLabel()==valve_status_ON:
            self.Valve25.SetLabel(valve_status_OFF)

        else:
            self.Valve25.SetLabel(valve_status_ON)

def V26(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve26.GetLabel()==valve_status_ON:
            self.Valve26.SetLabel(valve_status_OFF)

        else:
            self.Valve26.SetLabel(valve_status_ON)

def V27(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve27.GetLabel()==valve_status_ON:
            self.Valve27.SetLabel(valve_status_OFF)

        else:
            self.Valve27.SetLabel(valve_status_ON)

def V28(self, event):
        valve_status_ON = 'ON'
```

```
        valve_status_OFF = 'OFF'

        if self.Valve28.GetLabel()==valve_status_ON:
            self.Valve28.SetLabel(valve_status_OFF)

        else:
            self.Valve28.SetLabel(valve_status_ON)

    def V29(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve29.GetLabel()==valve_status_ON:
            self.Valve29.SetLabel(valve_status_OFF)

        else:
            self.Valve29.SetLabel(valve_status_ON)

    def V30(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve30.GetLabel()==valve_status_ON:
            self.Valve30.SetLabel(valve_status_OFF)

        else:
            self.Valve30.SetLabel(valve_status_ON)

    def V31(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'
```

```
        if self.Valve31.GetLabel()==valve_status_ON:
            self.Valve31.SetLabel(valve_status_OFF)

        else:
            self.Valve31.SetLabel(valve_status_ON)


    def V32(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve32.GetLabel()==valve_status_ON:
            self.Valve32.SetLabel(valve_status_OFF)

        else:
            self.Valve32.SetLabel(valve_status_ON)

    def V33(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve33.GetLabel()==valve_status_ON:
            self.Valve33.SetLabel(valve_status_OFF)

        else:
            self.Valve33.SetLabel(valve_status_ON)

    def V34(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve34.GetLabel()==valve_status_ON:
            self.Valve34.SetLabel(valve_status_OFF)
```

```
        else:
            self.Valve34.SetLabel(valve_status_ON)

    def V35(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve35.GetLabel()==valve_status_ON:
            self.Valve35.SetLabel(valve_status_OFF)

        else:
            self.Valve35.SetLabel(valve_status_ON)

    def V36(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve36.GetLabel()==valve_status_ON:
            self.Valve36.SetLabel(valve_status_OFF)

        else:
            self.Valve36.SetLabel(valve_status_ON)

    def V37(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve37.GetLabel()==valve_status_ON:
            self.Valve37.SetLabel(valve_status_OFF)

        else:
```

```
            self.Valve37.SetLabel(valve_status_ON)

    def V38(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve38.GetLabel()==valve_status_ON:
            self.Valve38.SetLabel(valve_status_OFF)

        else:
            self.Valve38.SetLabel(valve_status_ON)

    def V39(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve39.GetLabel()==valve_status_ON:
            self.Valve39.SetLabel(valve_status_OFF)

        else:
            self.Valve39.SetLabel(valve_status_ON)

    def V40(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve40.GetLabel()==valve_status_ON:
            self.Valve40.SetLabel(valve_status_OFF)

        else:
            self.Valve40.SetLabel(valve_status_ON)

    def V41(self, event):
```

```
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve41.GetLabel()==valve_status_ON:
            self.Valve41.SetLabel(valve_status_OFF)

        else:
            self.Valve41.SetLabel(valve_status_ON)

    def V42(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve42.GetLabel()==valve_status_ON:
            self.Valve42.SetLabel(valve_status_OFF)

        else:
            self.Valve42.SetLabel(valve_status_ON)

    def V43(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve43.GetLabel()==valve_status_ON:
            self.Valve43.SetLabel(valve_status_OFF)

        else:
            self.Valve43.SetLabel(valve_status_ON)

    def V44(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'
```

```
        if self.Valve44.GetLabel()==valve_status_ON:
            self.Valve44.SetLabel(valve_status_OFF)

        else:
            self.Valve44.SetLabel(valve_status_ON)

    def V45(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve45.GetLabel()==valve_status_ON:
            self.Valve45.SetLabel(valve_status_OFF)

        else:
            self.Valve45.SetLabel(valve_status_ON)

    def V46(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve46.GetLabel()==valve_status_ON:
            self.Valve46.SetLabel(valve_status_OFF)

        else:
            self.Valve46.SetLabel(valve_status_ON)

    def V47(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve47.GetLabel()==valve_status_ON:
            self.Valve47.SetLabel(valve_status_OFF)
```

```
        else:
            self.Valve47.SetLabel(valve_status_ON)

    def V48(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve48.GetLabel()==valve_status_ON:
            self.Valve48.SetLabel(valve_status_OFF)

        else:
            self.Valve48.SetLabel(valve_status_ON)

    def V49(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve49.GetLabel()==valve_status_ON:
            self.Valve49.SetLabel(valve_status_OFF)

        else:
            self.Valve49.SetLabel(valve_status_ON)

    def V50(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve50.GetLabel()==valve_status_ON:
            self.Valve50.SetLabel(valve_status_OFF)

        else:
```

```
            self.Valve50.SetLabel(valve_status_ON)

    def V51(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve51.GetLabel()==valve_status_ON:
            self.Valve51.SetLabel(valve_status_OFF)

        else:
            self.Valve51.SetLabel(valve_status_ON)

    def V52(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve52.GetLabel()==valve_status_ON:
            self.Valve52.SetLabel(valve_status_OFF)

        else:
            self.Valve52.SetLabel(valve_status_ON)

    def V53(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve53.GetLabel()==valve_status_ON:
            self.Valve53.SetLabel(valve_status_OFF)

        else:
            self.Valve53.SetLabel(valve_status_ON)

    def V54(self, event):
```

```
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve54.GetLabel()==valve_status_ON:
            self.Valve54.SetLabel(valve_status_OFF)

        else:
            self.Valve54.SetLabel(valve_status_ON)

    def V55(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve55.GetLabel()==valve_status_ON:
            self.Valve55.SetLabel(valve_status_OFF)

        else:
            self.Valve55.SetLabel(valve_status_ON)

    def V56(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve56.GetLabel()==valve_status_ON:
            self.Valve56.SetLabel(valve_status_OFF)

        else:
            self.Valve56.SetLabel(valve_status_ON)

    def V57(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'
```

```
        if self.Valve57.GetLabel()==valve_status_ON:
            self.Valve57.SetLabel(valve_status_OFF)

        else:
            self.Valve57.SetLabel(valve_status_ON)

    def V58(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve58.GetLabel()==valve_status_ON:
            self.Valve58.SetLabel(valve_status_OFF)

        else:
            self.Valve58.SetLabel(valve_status_ON)

    def V59(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve59.GetLabel()==valve_status_ON:
            self.Valve59.SetLabel(valve_status_OFF)

        else:
            self.Valve59.SetLabel(valve_status_ON)

    def V60(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve60.GetLabel()==valve_status_ON:
            self.Valve60.SetLabel(valve_status_OFF)
```

```
        else:
            self.Valve60.SetLabel(valve_status_ON)

    def V61(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve60.GetLabel()==valve_status_ON:
            self.Valve60.SetLabel(valve_status_OFF)

        else:
            self.Valve60.SetLabel(valve_status_ON)

    def V62(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve60.GetLabel()==valve_status_ON:
            self.Valve60.SetLabel(valve_status_OFF)

        else:
            self.Valve60.SetLabel(valve_status_ON)

    def V63(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve60.GetLabel()==valve_status_ON:
            self.Valve60.SetLabel(valve_status_OFF)

        else:
```

```
            self.Valve60.SetLabel(valve_status_ON)



    def V64(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve60.GetLabel()==valve_status_ON:
            self.Valve60.SetLabel(valve_status_OFF)

        else:
            self.Valve60.SetLabel(valve_status_ON)



    def V65(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve60.GetLabel()==valve_status_ON:
            self.Valve60.SetLabel(valve_status_OFF)

        else:
            self.Valve60.SetLabel(valve_status_ON)

    def V66(self, event):
        valve_status_ON = 'ON'
        valve_status_OFF = 'OFF'

        if self.Valve60.GetLabel()==valve_status_ON:
            self.Valve60.SetLabel(valve_status_OFF)
```

```
        else:
            self.Valve60.SetLabel(valve_status_ON)

#*********************          Define Pumps          ********************
#*****************************************************************************************

    def P1 (self,event):

        wx.MessageBox("P1 is Open ","Pump", wx.OK|wx.ICON_INFORMATION)

    def P2 (self,event):
        wx.MessageBox("P2 is Open ","Pump", wx.OK|wx.ICON_INFORMATION)

    def P3 (self,event):
        wx.MessageBox("P3 is Open ","Pump", wx.OK|wx.ICON_INFORMATION)

    def P4 (self,event):
        wx.MessageBox("P4 is Open ","Pump", wx.OK|wx.ICON_INFORMATION)

    def P5 (self,event):
        wx.MessageBox("P5 is Open ","Pump", wx.OK|wx.ICON_INFORMATION)

    def P6 (self,event):
        wx.MessageBox("P6 is Open ","Pump", wx.OK|wx.ICON_INFORMATION)

    def P7 (self,event):
        wx.MessageBox("P7 is Open ","Pump", wx.OK|wx.ICON_INFORMATION)

    def P8 (self,event):
        wx.MessageBox("P8 is Open ","Pump", wx.OK|wx.ICON_INFORMATION)

    def P9 (self,event):
```

```
        wx.MessageBox("P9 is Open ","Pump", wx.OK|wx.ICON_INFORMATION)


    def P10 (self,event):
        wx.MessageBox("P10 is Open ","Pump", wx.OK|wx.ICON_INFORMATION)




    ##########################################################################################




#**********************          listbox          **********************

#*****************************************************************************************
```

```
class ValveListBox(wx.Frame):

 def __init__(self):

    wx.Frame.__init__(self, None, -1,'Pumps list', size=(250,300))

    panel = wx.Panel(self,-1)

    #List1=Listbox()
    #List1.insert(1,'P1')
    #List1.insert(2,'P2')
```

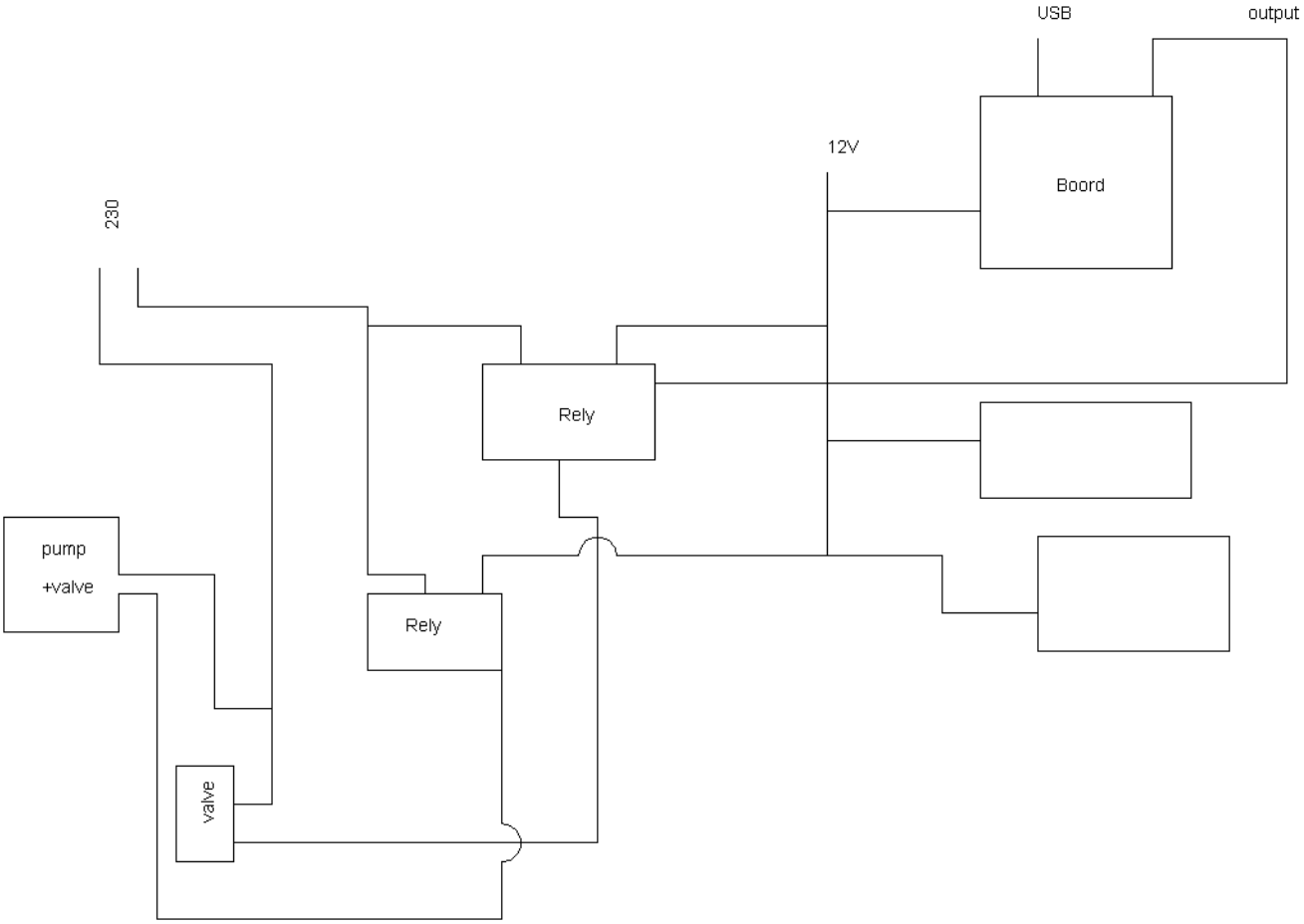
```
    #List1.insert(3,'P3')
    #List1.insert(4,'P4')
    #List1.insert(5,'P5')
    #List1.insert(6,'P6')
    #List1.insert(7,'P7')
    #List1.insert(8,'P8')
    #List1.insert(9,'P9')
   # List1.insert(10,'P10')
    LABELSTYLE = wx.BORDER_SUNKEN | wx.ST_NO_AUTORESIZE | wx.ALIGN_CENTER_HORIZONTAL


    wx.StaticText(panel,20," Pumps Selected : ",(10,10))
    wx.StaticText(panel,-1," P1 ",(18,40))
    wx.StaticText(panel,-1," P2 ",(18,70))
    wx.StaticText(panel,-1," P3 ",(18,100))
    wx.StaticText(panel,-1," P4 ",(18,130))
    wx.StaticText(panel,-1," P5 ",(18,160))
    wx.StaticText(panel,-1," P6 ",(18,190))
    wx.StaticText(panel,-1," P7 ",(18,220))
    wx.StaticText(panel,-1," P8 ",(18,250))

    wx.StaticText(panel,-1," P9 ",(18,280))
    wx.StaticText(panel,-1," P10 ",(18,310))

    self.Pump1 = wx.StaticText(panel, size = (40, -1), pos = (60, 40), style = LABELSTYLE)
    self.Pump2 = wx.StaticText(panel, size = (40, -1), pos = (60, 70), style = LABELSTYLE)
    self.Pump3 = wx.StaticText(panel, size = (40, -1), pos = (60, 100), style = LABELSTYLE)
    self.Pump4 = wx.StaticText(panel, size = (40, -1), pos = (60, 130), style = LABELSTYLE)
    self.Pump5 = wx.StaticText(panel, size = (40, -1), pos = (60, 160), style = LABELSTYLE)
    self.Pump6 = wx.StaticText(panel, size = (40, -1), pos = (60, 190), style = LABELSTYLE)
    self.Pump7 = wx.StaticText(panel, size = (40, -1), pos = (60, 220), style = LABELSTYLE)
    self.Pump8 = wx.StaticText(panel, size = (40, -1), pos = (60, 250), style = LABELSTYLE)
```

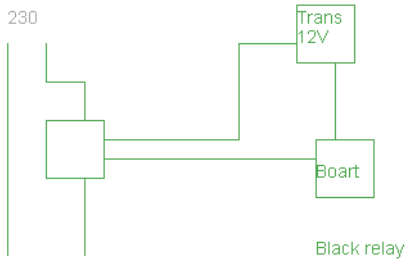
```
    self.Pump9 = wx.StaticText(panel, size = (40, -1), pos = (60, 280), style = LABELSTYLE)
    self.Pump10 = wx.StaticText(panel, size = (40, -1), pos = (60, 310), style = LABELSTYLE)









  #sampleList= ['P1','P2','P3','P4','P5','P6','P7','P8','P9','P10']
    #
'V21','V22','V23','V24','V25','V26','V27','V28','V29','V30','V31','V32','V33','V34','V35','V36','V37','V38','
V39','V40',
     #          'V41','V42','V43','V44','V45','V46','V47','V48']
       #wx.StaticText(panel,-1,"Pumps",(20,5))
       #wx.ComboBox(panel,-1," valve ",(20,30),wx.DefaultSize,sampleList,wx.CB_DROPDOWN)
      #wx.ComboBox(panel,-1," valve ",(150,30),wx.DefaultSize,sampleList,wx.CB_SIMPLE)


  # listBox = wx.ListBox(panel, -1, (0, 0), (250, 300), sampleList,wx.LB_EXTENDED)
  # listBox.SetSelection(3)


  # listbox = wx.CheckListBox(panel,-1,(20,20),(100,300),sampleList, wx.LB_SINGLE)
    def P1 (self,event):


          pump_status_ON = 'ON'
          pump_status_OFF = 'OFF'


          if self.Pump1.GetLabel()==pump_status_ON:
             self.Pump1.SetLabel(pump_status_OFF)


          else:
             self.Pump1.SetLabel(pump_status_ON)
```

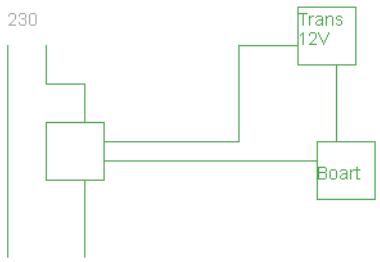
```
        wx.MessageBox("P1 is Open ","Pump", wx.OK|wx.ICON_INFORMATION)

    def P2 (self,event):
        wx.MessageBox("P2 is Open ","Pump", wx.OK|wx.ICON_INFORMATION)

    def P3 (self,event):
        wx.MessageBox("P3 is Open ","Pump", wx.OK|wx.ICON_INFORMATION)

    def P4 (self,event):
        wx.MessageBox("P4 is Open ","Pump", wx.OK|wx.ICON_INFORMATION)

    def P5 (self,event):
        wx.MessageBox("P5 is Open ","Pump", wx.OK|wx.ICON_INFORMATION)

    def P6 (self,event):
        wx.MessageBox("P6 is Open ","Pump", wx.OK|wx.ICON_INFORMATION)

    def P7 (self,event):
        wx.MessageBox("P7 is Open ","Pump", wx.OK|wx.ICON_INFORMATION)

    def P8 (self,event):
        wx.MessageBox("P8 is Open ","Pump", wx.OK|wx.ICON_INFORMATION)

    def P9 (self,event):
        wx.MessageBox("P9 is Open ","Pump", wx.OK|wx.ICON_INFORMATION)

    def P10 (self,event):
        wx.MessageBox("P10 is Open ","Pump", wx.OK|wx.ICON_INFORMATION)
```

```
#*********************        main definition and loop        ******************
#*****************************************************************************************

def main():
    """Testing"""
    MyBackgroundPanel = wx.PySimpleApp(wx.Panel)
    f = MyFrame()

#in D:\AECENAR\MEGBI\MEGBI-VPP\AUT\CDMasterThesisHayssamHindy\GUI\101005_MEGBI-VPP_downstream.py the follwowing two lines are not in comment
    #d=ValveListBox()
    #d.Show()
    f.Center()
    f.Show()
    MyBackgroundPanel.MainLoop()

if __name__ == "__main__":
    main()
```

19.5 Annex 3: Electrical circuits for connection between veleman board, relais and actuators (pump. valve)







Rough Business Plans and Commercializing Market Strategy

20 Project Management / إدارة المشروع

20.1 Project goal /هدف /العمل

The goal is to install a DNA vaccine production plant.

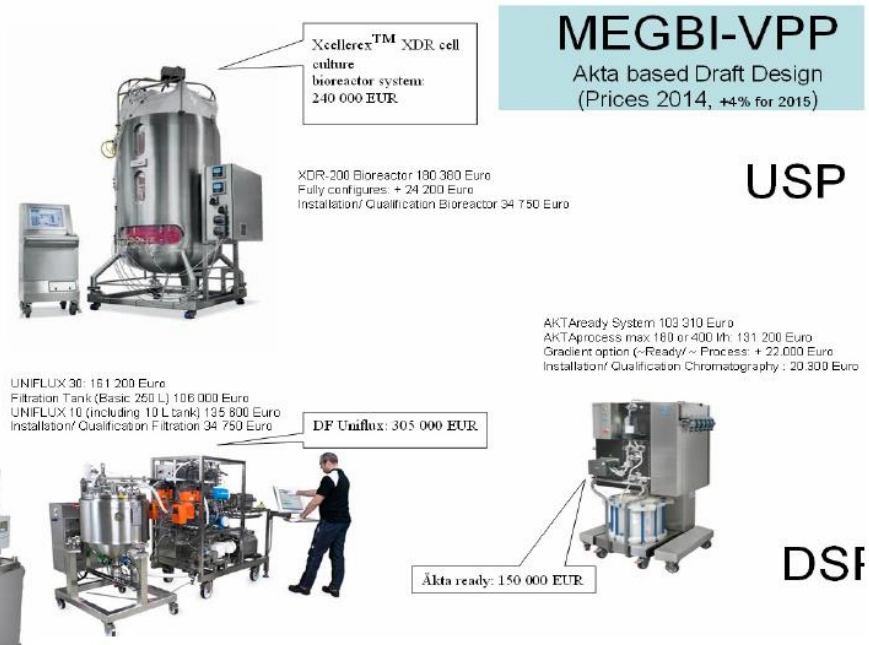
1. HBSAg vaccine production based on S.cerevisae
2. later on migration to MAB production in E.coli

20.2 Budget Planning

20.2.1 Akta Based Plant

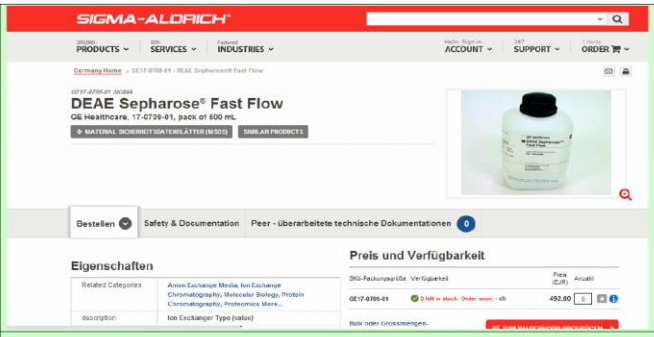
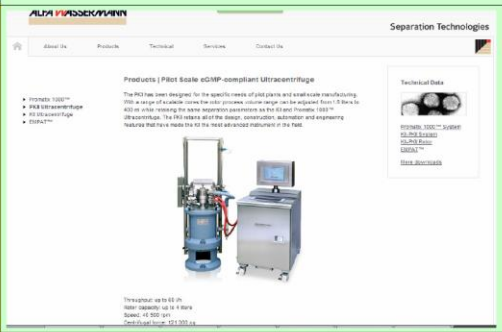
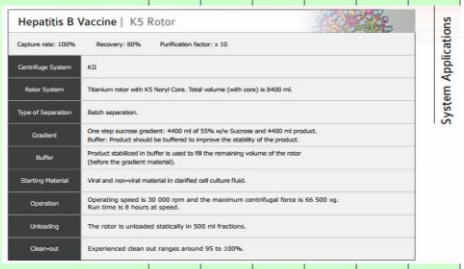
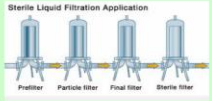
Smaller Scale	Prices 2014	Prices 2015 including transport and douane in Lebanon
Bioreactor (offer from 2013)	130.000 €	175.760 €
Uniflux (Filtration) 10	172.000 €	232.544 €
Aktaready	140.000 €	189.280 €
Ultracentrifuge	50.000 €	67.600 €
DNA Laboratory		130.000 €
Total		**795.184 €**

Medium Scale	Prices 2014	Prices 2015 including transport and douane in Lebanon
Bioreactor Xcell	240.000 €	324.480 €
Uniflux (Filtration) 30	305.000 €	412.360 €
AktaProcess	170.000 €	229.840 €
Ultracentrifuge	50.000 €	67.600 €
DNA Laboratory		130.000 €
Total		**1.164.280 €**



20.2.2 Patent Based semi-own manufactoring

Last update: 24.12.2014

Device No.	Device Name	Price including transport & customs	Supplier/image
1	Disc Stack Centrifuge 1	$25.000	
2	Homogenizer	$15.000	
3	Storage Tank/ PEG Precipitation mixing tank 100L	$2.000	
4	Disc Stack Centrifuge 2	$25.000	
5	Storage Tank/ CaCl2 Precipitation mixing tank 100L	$2.000	
6	Ultrafiltration Device	$5.000	
7	Storage Tank 100L for UF	$2.000	
8	20 L Chromatography column	$3.000	
9	20 L Sepharose 4B	$18.000	
10	Storage Tank 100L	$2.000	
11	20 L Chromatography column	$3.000	
12	10 L DEAE matrix DFF100 DEAE–Sepharose® Fast Flow (Sigma Aldrich)	$17.000	
13	Storage Tank 100L	$2.000	
14	Ultracentrifugation CaCl	$150.000	 
15	20 L Chromatography column	$3.000	
16	20 L Sepharose 4BCLFF	$18.000	
17	Storage Tank 100L	$2.000	
18	sterile filtration 0.22 Mikrometer	$2.500	
19	Chemicals	5000	
20	Engineering 6 man months	18000	
21	Project Management 6 man months	24000	
	Total	**$343.500**	

20.2.3 Budget for MEGBI Vaccine Production Pilot Plant (MEGBI-VPP)

MEGBI-VPP
DNA Vaccine Pilot Plant for production of Hepatitis B virus
2015

Last Update: 04. Feb 15

DNA Transfer Laboratory

device	price
Safety Cabinet B2	8.000 €
centrifuge	5.000 €
Shake incubator	2.500 €
Thermocycler	2.500 €
blood freezer	3.500 €
Gel rack with voltage	1.500 €
Precision Balance	800 €
Minus 80 °C freezer	10.000 €
UV Machine	1.100 €
vortex	300 €
precision pipettes	500 €
molecular biological reagences	4.000 €
chemicals	2.000 €
laboratory materials	1.500 €
laboratory tables	1.500 €
IT	1.000 €
fluorescence microscope	3.000 €

Transportation and customs	6.000 €
Building Lab 2009-11	25.000 €
Total Laboratory	**79.700 €**

Upstream Processing (Bioreactor)

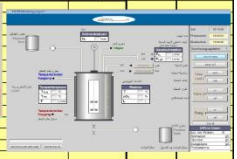
Material Costs

System	Piece	#	Piece price	Total
Behälter (130l Stainless)		1	600 €	600 €
Temperiersystem	Behälter	2	50 €	100 €
	Abdeckwolle	1	100 €	100 €
	Blech	1	120 €	120 €
	Pumpe	1	80 €	80 €
	Beheizungsrad	1	60 €	60 €
Aut. Valve		2	200 €	400 €
Temp.Sensor		1	20 €	20 €
PH Sensor		1	100 €	100 €
PO_2 Sensor		1	1.200 €	1.200 €
Ausfluß	Aut.Valve	1	200 €	200 €
Medium Einfluß	Aut.Valve	1	200 €	200 €
	Behälter	1	50 €	50 €
PH Regulierung	Behälter	2	50 €	100 €
	Aut.Valve	2	200 €	400 €
Beimpfungsreinigung	Behälter	1	50 €	50 €
	Aut.Valve	1	200 €	200 €
Control System	Siemens S7 PLC	1	1.500 €	1.500 €
	PC	1	500 €	500 €
			Sum Material	5.980 €

Personnel Costs

Task	duration (months)	Qualification	price / month	Total
Integration Mechanics	1	Eng.	2.000 €	2.000 €
Integration Automation System	1	Eng.	2.000 €	2.000 €
Programming Control AECENAR	1	Eng.	2.000 €	2.000 €
Project Managing	3	Eng.	2.000 €	6.000 €
			Sum Personnel Costs	12.000 €

Personal Specific Costs	cost per month
Student	0 €
Specialized Worker	1.000 €
Engineer	2.000 €



Total Upstream Processing Unit (Bioreactor)	**17.980 €**

Downstream Processing

	price per piece	pieces needed	Costs	Needed Staff
Homogenizer	1.000 €	1	1.000 €	
Prototype Chromatogr. Process Device (mech)	13.000 €	3	39.000 €	
Prototype Chromatogr. Process Device(autom)			0 €	
Ultrafiltration Unit	1.500 €	1	1.500 €	
Ultracentrifuge (used)			0 €	
Disc Stack Centrifuge	10.500 €	1	10.500 €	
Mixing/Storing tanks	1.000 €	4	4.000 €	
Pump	700 €	6	4.200 €	
Automation (PLC system, GUI)			10.000 €	
Total Devices	73.200 €	Integration (piping, ...)	3.000 €	**Total Downstream Processing Unit** **76.200 €**

Project Management

		#months	monthly rate for manage-ment	
Program Managing over 9 months	9		2.000,00 €	18.000 €
Documentation				4.000 €
				Total Project Management **22.000 €**

Total MEGBI-VPP	**195.880 €**

planned project duration: Dez. 2014 - August 2015

MEGBI-VPP
DNA Vaccine Pilot Plant for production of Hepatitis B virus
2015

Last Update: 04. Feb 15

DNA Transfer Laboratory

device	price
Safety Cabinet B2	8.000 €
centrifuge	5.000 €
Shake incubator	2.500 €
Thermocycler	2.500 €
blood freezer	3.500 €
Gel rack with voltage	1.500 €
Precision Balance	800 €
Minus 80 °C freezer	10.000 €
UV Machine	1.100 €
vortex	300 €
precision pipettes	500 €
molecular biological reagences	4.000 €
chemicals	2.000 €
laboratory materials	1.500 €
laboratory tables	1.500 €
IT	1.000 €
fluorescence microscope	3.000 €

Transportation and customs	6.000 €
Building Lab 2009-11	25.000 €
Total Laboratory	**79.700 €**

Upstream Processing (Bioreactor)

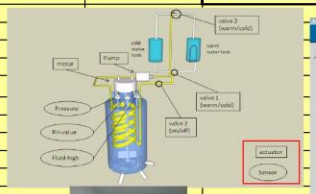
Material Costs

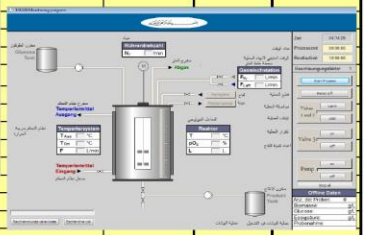
System	Piece	#	Piece price	Total
Behälter (130l Stainless)		1	600 €	600 €
Temperiersystem	Behälter	2	50 €	100 €
	Abdeckwolle	1	100 €	100 €
	Blech	1	120 €	120 €
	Pumpe	1	80 €	80 €
	Beheizungsrad	1	60 €	60 €
Aut. Valve		2	200 €	400 €
Temp.Sensor		1	20 €	20 €
PH Sensor		1	100 €	100 €
PO_2 Sensor		1	1.200 €	1.200 €
Ausfluß	Aut.Valve	1	200 €	200 €
Medium Einfluß	Aut.Valve	1	200 €	200 €
	Behälter	1	50 €	50 €
PH Regulierung	Behälter	2	50 €	100 €
	Aut.Valve	2	200 €	400 €
Beimpfungsreinigung	Behälter	1	50 €	50 €
	Aut.Valve	1	200 €	200 €
Control System	Siemens S7 PLC	1	1.500 €	1.500 €
	PC	1	500 €	500 €
			Sum Material	5.980 €

Personnel Costs

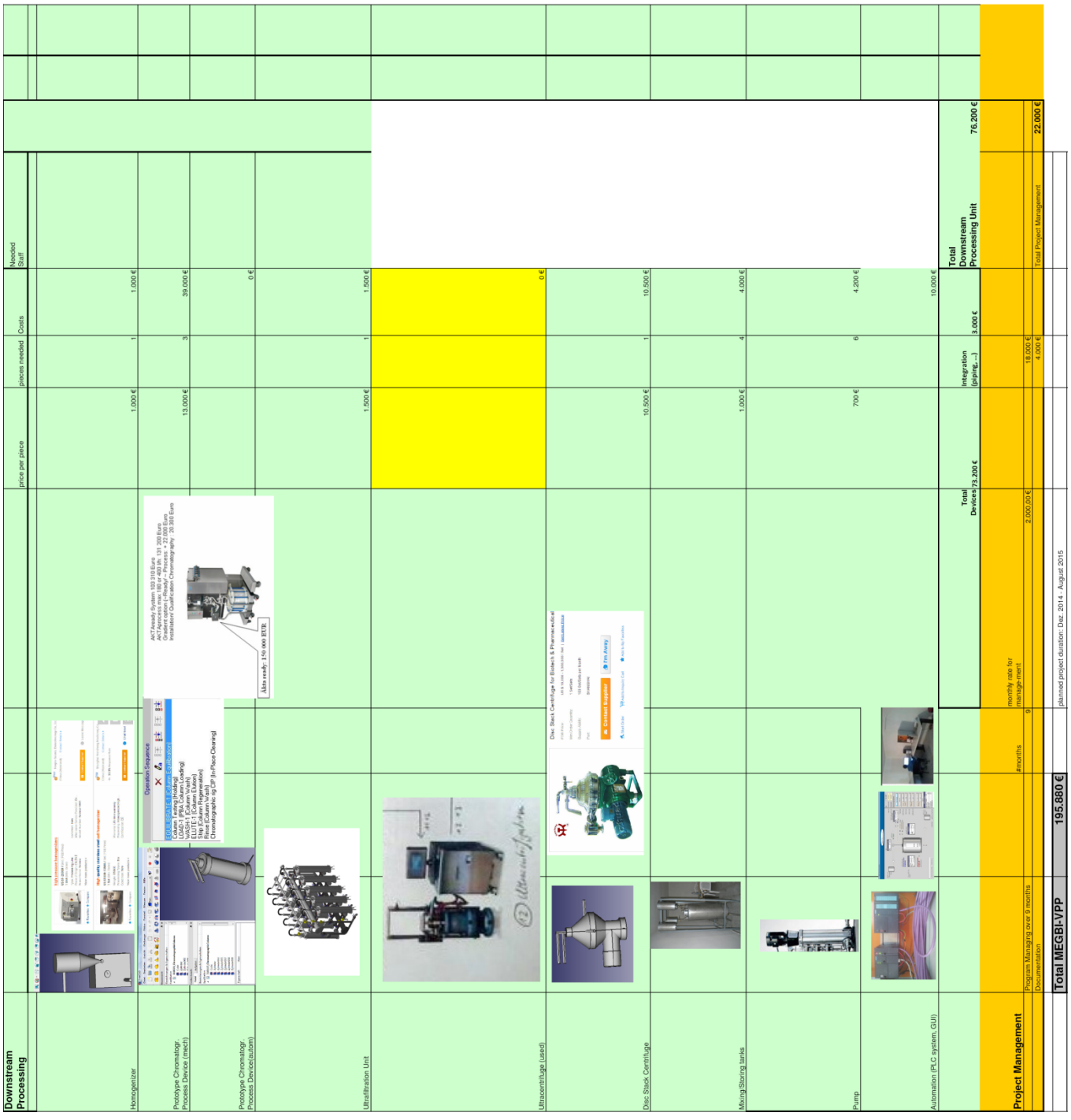
Task	duration (months)	Qualification	price / month	Total
Integration Mechanics	1	Eng.	2.000 €	2.000 €
Integration Automation System	1	Eng.	2.000 €	2.000 €
Programming Control	1	Eng.	2.000 €	2.000 €
AECENAR Project Managing	3	Eng.	2.000 €	6.000 €
			Sum Personnel Costs	**12.000 €**

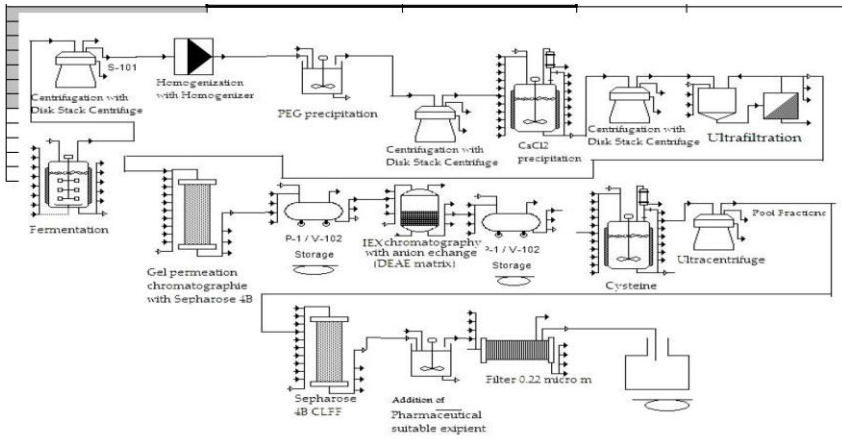
Personal Specific Costs	cost per month
Student	0 €
Specialized Worker	1.000 €
Engineer	2.000 €





Total Upstream Processing Unit (Bioreactor)	**17.980 €**


Downstream Processing
Needed Staff
price per piece
pieces needed
Costs
Homogenizer
1.000 €
1
1.000 €
Prototype Chromatogr. Process Device (mech)
13.000 €
3
39.000 €
Prototype Chromatogr. Process Device (fluidic)
0 €
Ultrafiltration Unit
1.500 €
1
1.500 €
0 €
Ultracentrifuge (used)
10.500 €
1
10.500 €
Disc Stack Centrifuge
1.000 €
4
4.000 €
Mixing/Storing tanks
700 €
6
4.200 €
Pump
10.000 €
Automation (PLC system, GUI)
Total Devices 73.200 €
Integration (piping, ...) 3.000 €
Total Downstream Processing Unit 76.200 €
Project Management
#months
monthly rate for manage-ment
2.000,00 €
18.000 €
Program Managing over 9 months
9
Documentation
4.000 €
Total Project Management 22.000 €
Total MEGBI-VPP 195.880 €
planned project duration: Dez. 2014 - August 2015



Clean-in-place (CIP)&Steam-in-place (SIP) and intermediate storage	Upstream Processing (USP) – Downstream Processing (DSP)	Automation
 		   

Project Status Feb 2015

so far completed	67%	
still open	33%	**64.640 €**

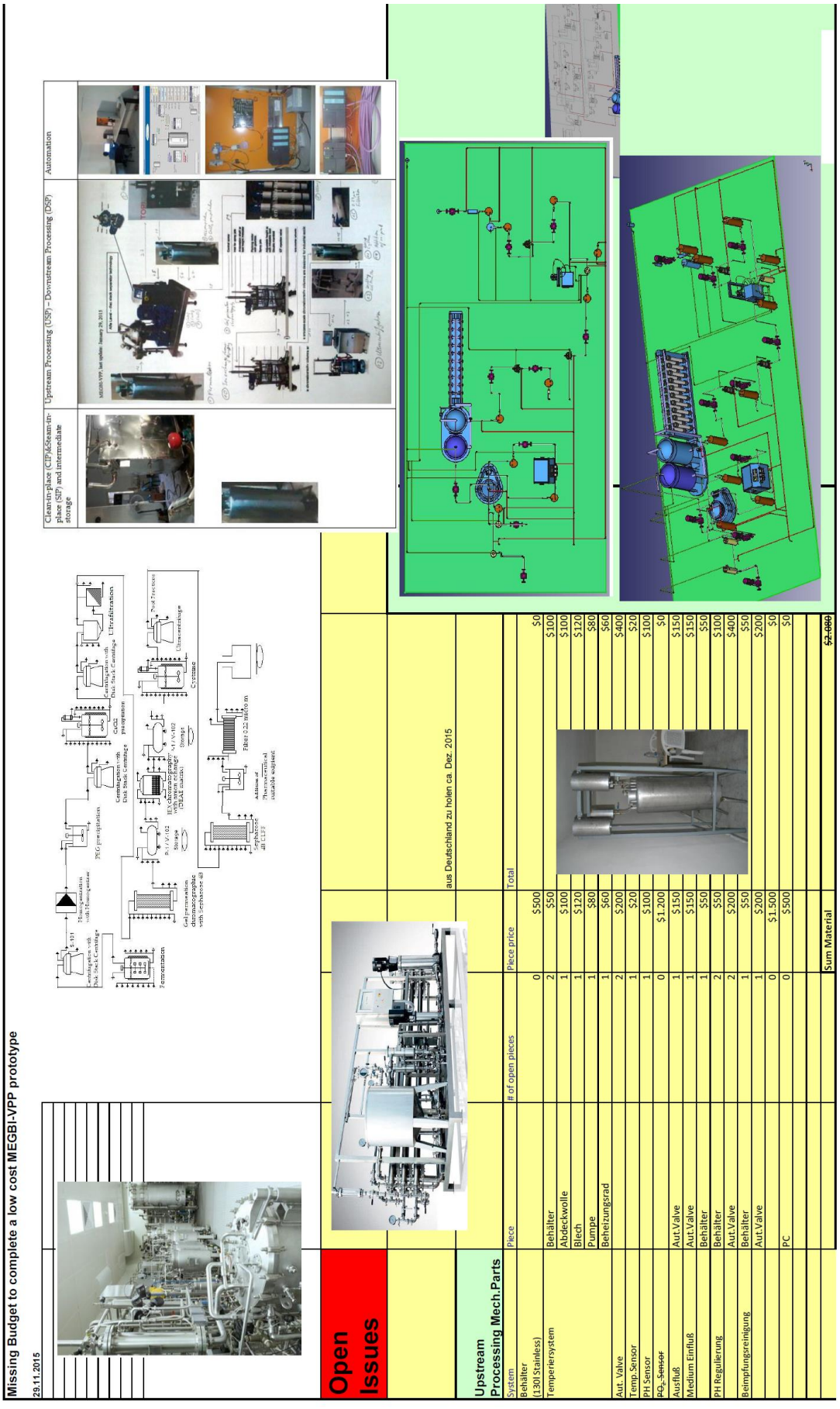
required financing to complete the pilot plant

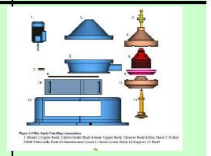
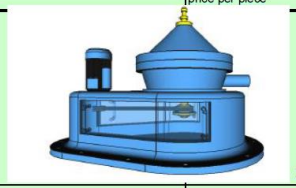
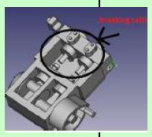
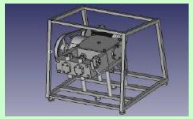
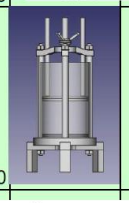
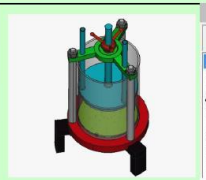
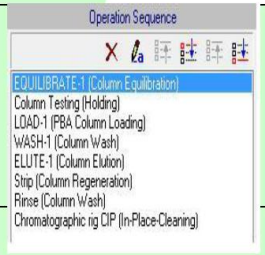
Strategy for Market and Industrial Infrastructure Building in North Lebanon

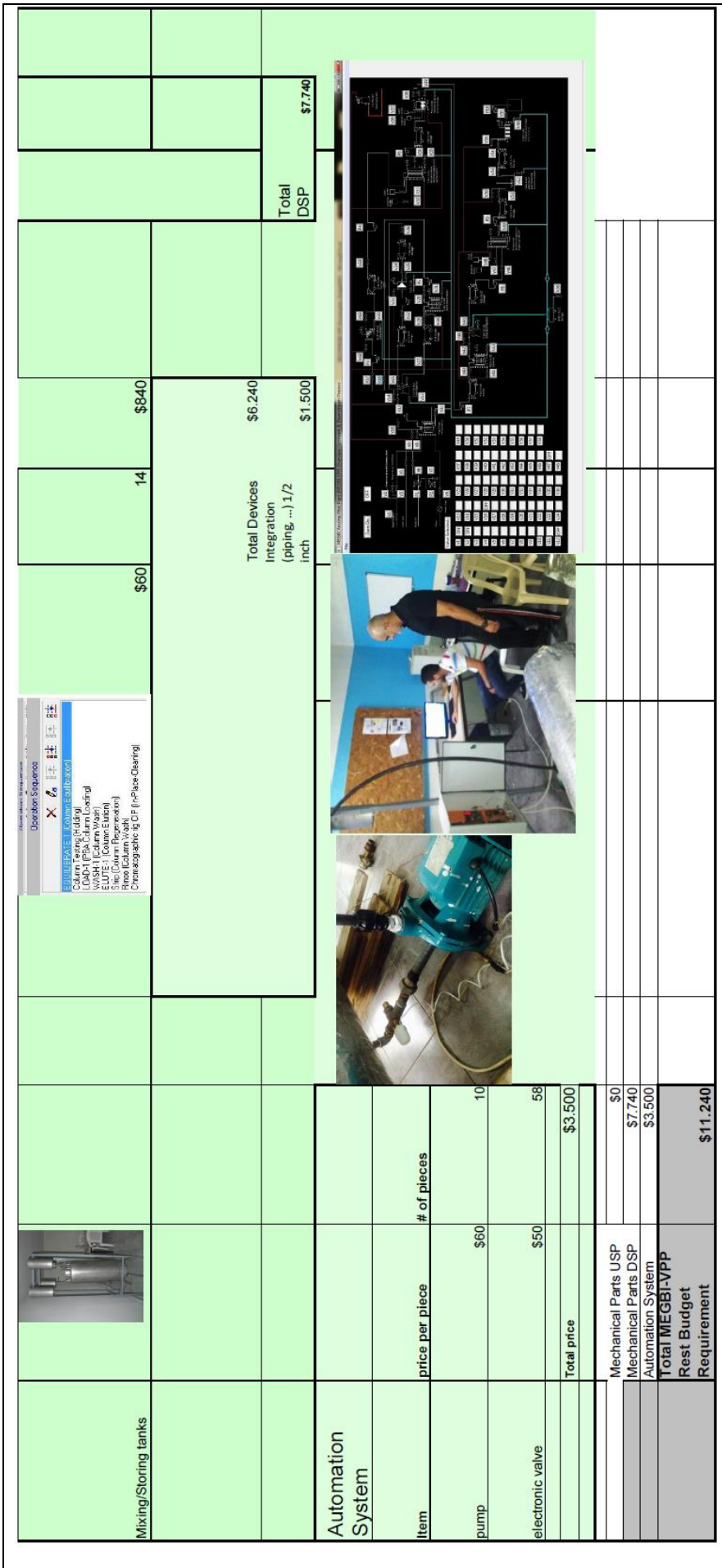
2015	2016	2017	2018	2019	2020	2021
MEGBI-VPP		MAB financed	market access	supplier startup companies		
DNA vaccine pilot plant			producing MAB (monoclonal antibodies)			

This calculation was presented to LASeR (Prof Moustafa Jazar, Dr Ammar Assoum, Dr Bachar al-Hasan) and discussed at LU Doctoral School in Tripoli (Dr Ammar Assoum, Dr Bachar al-Hasan, Dr Mohammad Khalil). Result: LASeR waits for the opinion of Prof Monzer Hamze (Status 17 March 2015), one phone call with Dr Monzer in March 15, then no further anwers).

20.2.4 Budget Plan for completing a minimal Prototype Plan (Status November 15)



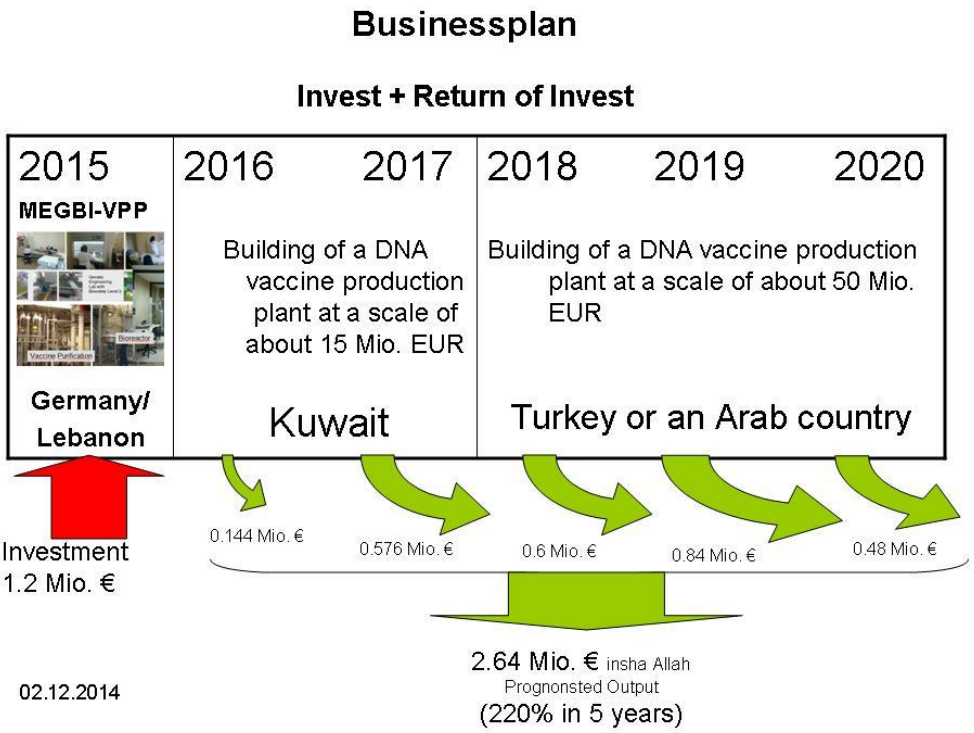
Downstream Processing	# of mechnical pieces	average complexity/cost of part			price per piece	pieces needed	Costs	Needed Staff			
Disc Stack Centrifuge	20	$70			$1.400	1	$1.400				
Homogenizer	10	$120			$1.200	1	$1.200				
Chromatogr. Column (mech)	8	$50			$400	3	$1.200				
Ultrafiltration Unit					$200	1	$200				
Ultracentrifuge (used)	20	$70			$1.400	1	$1.400				



This version includes:

- smaller vessels
- simple disc stack centrifuge
- cost reduction option: vessel with chemical opening of cells instead of homogenizer
- disc stack centrifuge instead of ultracentifuge
- main new manufactoring: chromatographic columns (requires fine mechanical manufactoring facility)

20.3 Business Plan 1 (presented Dec 2014)



20.3.1 Investors for Business Plan 1

Übersicht der Investoren am MEGBI-VPP

Stand: **31.12.2014**

Investoren

Gesamtentwicklungswert **1.200.000 €**

Investor	Höhe des Investitionswertes	Anteile am Gewinn (Entwicklung) bis April 2011	Bemerkung/Datum der Investition
Amine Bouafif	100,25 €	0,0083542%	Investion bezahlt (Überweisung ca. 11.12.14)
Nasser Al Araimi	1.200 €	0,1000000%	Investition bezahlt (Überweisung 27.12.14)
David Yildiz	600 €	0,0500000%	Investion bezahlt (bar ca. 8.12.14)
AECENAR	133.000 €	11,0833333%	DNA Labor 130TEUR, Miete Jan-Jun 15 3TEUR
Summe:	134.900 €	11,2416875%	
Restentwicklungsanteile TEMO	1.065.100 €	88,76%	

derzeit ist der größte Teil der Projektdokumente öffentlich zugänglich und hier einsehbar:

http://temo-ek.de/8.html

20.3.2 Location Concept of Business Plan 1

Cooperation between Europe and the Middle East

By the cooperation with Turkish and Arab partners the MEGBI-VPP project creates working possibilities for the young educated people in the Middle East region and helps to stabilize the region.

And with the help of God, the Almighty, this will be a big effort for a better and peaceful future for the two neighbour regions Europe and North-Africa/Middle East.

The genetic engineering laboratory of the pilot plant (for step 1) is based in North Lebanon at the nice village Ras Nhache. Threre were also undergone the prestudies of the project.

Bioreactor (step 2) and purification (step 3) will be implemented at TEMO Biotechnology site at Heidelberg, Germany

The administration of the project will be done by TEMO Biotechnology in Germany.

Software, Hardware & Consulting e.K. – TEMO

TEMO Biotechnology
TEMO e.K.
Im Klingenbühl 2a
69123 Heidelberg, Germany

Email: info@temo-ek.de
Website: www.temo-ek.de

20.4 Business Plan 2 (Presented Feb 2015 to Azm Association)

Bismillah

MEGBI-VPP Businessplan and Feasibility Study

18.2.2015

1. The Goal: a Facility able to produce vaccines and antibodies based on fermentation and purification (biotechnological upstream and downstream process)

The Goal is a company like this

Facility History

- **1988 -** Cetus Corporation, Emeryville, CA, US establishes its European headquarters, EuroCetus B.V. in Amsterdam, the Netherlands.
- **1990-1991 -** EuroCetus facility licensed for production and release of ProLeukin (IL-2) for Europe and rest of world, except US and Japan.
- **1992 –** Chiron Corporation acquires Cetus Corporation. Eurocetus B.V. becomes Chiron B.V.
- **1996 –** Facility licensed for commercial production of Pertussis vaccine.
- **1997 –** Facility licensed by WHO for commercial production of Meningitis A polysaccharides.
- **1997-1999 –** Facility licensed by UK, MCA and Italian Health Branch for Meningitis C polysaccharide and CRM197 carrier protein for conjugated vaccine Menjugate®.
- **2000 –** Facility acquired by Dr William J. Rutter. SynCo Bio Partners B.V. established.
- **2001 –** Facility licensed by EMA and Health Canada for Meningitis C polysaccharides and CRM197 carrier protein for Menjugate® production.
- **2004 –** Installation of new air handling systems completed for all of its facilities.
- **2005 –** Installation of new aseptic filling machine in its Class A zone, allowing larger batches sizes and expansion of filling ranges.
- **2005 –** Expansion of GMP facilities to allow production of a wider range of protein, vaccine and live biotherapeutic products.
- **2006 –** Facility licensed by Korean FDA for commercial production.

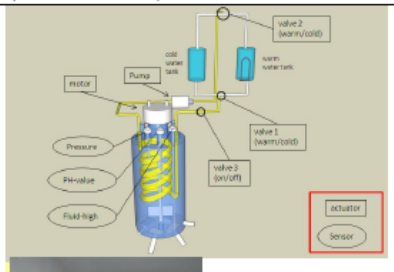
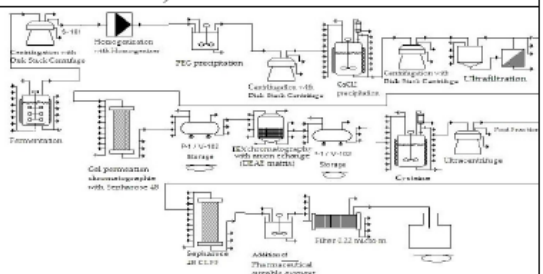
- **2008 –** Expansion of process development capabilities to enable a greater range of projects to be completed.
- **2009 –** Facility licensed by ANVISA for commercial production.
- **2011 –** Expansion of the Class A zone of its aseptic filling facility successfully completed, allowing SynCo to support new product launches in the US and the rest of the world.

2. Business Plan for our plant

Year	2009-2015	2016	2017	2018
	MEGBI Vaccine Production Pilot Plant (MEGBI-VPP)		Licenced for production of diagnostic antibodies (diagnosis of blood groups) in Lebanon/Jordan/Turkey and other Arab Countries	Licenced for production of Hepatitis B antigen vaccine (similar to Engerix) in Lebanon and other Arab Countries
Investment Costs	**120,000 € (still open to complete: 65.000 EUR)**	**4 Mio. EUR**		
Return of Invest			About 20 x 250 blood laboratories&blood banks: 2017-2019 5000x40x12 EUR = 2.4 Mio. EUR income per year **7.2 Mio. EUR**	

2.1. 2009-2015

MEGBI-VPP pilot plant shall convince an investor to invest in the next steps

Genetic Engineering Laboratory	Upstream Processing (Bioreactor)	Downstream Processing Unit (Protein Purification)
		
		

2.2. 2016

A plant like this:

Azm Association (Faisal Maulawi, Dr Dani Saaduddin, Dr Kifah Tout) visited AECENAR Center at Ras Nhache on 6th March 2015 and Business Plan 2 was discussed. Result (Status 17th March 2015): Azm wants a more detailed business plan with detailed market strategy.

20.5 Time Schedule / الجدول الزمني/

Nov/Dec 14: Financement and Concept Phase

Jan – June 15: Finishing of Development of MEGBI Vaccine Production Pilot Plant (MEGBI-VPP)

	Planned	Staff
System Design with SuperPro	15.12.-24.12.2014	
Automation System (GUI, veleman board)	1.3.-15.10.2015	Haitham Hindi (Master Student)
Design of mechanical parts with FreeCAD (Vessels, Storage Tanks, columns, Disc Stack Centrifuge, Ultracentrifuge, Homogenizer)	Mai - July 2015	Practicants from BAU (Jihad, Zaher, Ibrahim, Fadi)
Materials List (sensors, actuators, chemicals to be purchased)		
Manufactoring a low cost version of MEGBI-VPP (	16.3.-30.7.15	

مراجع (References)

20.6 IGEEH/MEGBI Research Reports

[Gafsi 2005] Diplomarbeit

[MEGBI-H5N1 2011] Abdulwahab N., Mourad S., Borghol S., Laboratory verifiying of the peptid candidate FLKDVMESM by ELISA and IFN-γ ELISPOT analysis (Step 2 in MEGBI H5N1 peptid vaccine research project), Ras Nhache, June 2011 (engl./عربي)

[MEGBI-VPP 2012] Samir Mourad, Rihab El Merheb, Layal Chbib, *" MEGBI Vaccine Pilot Plant – 1st Project Report (Feb 2012 – Jan 2013)", Introduction to Biotechnological upstream and downstream processing*

مدخل تطبيقي الى البيوتكنولوجيا، **تكثير خلايا في داخلها جينات يراد انتاج بروتينها وتنقية هذه البروتيئينات**

جميع التفاصيل باللغة العربية والمصطلحات العلمية باللغتين العربية والانجليزية

[MEGBI-VPP 2013] Samir Mourad, Jawdat Al Khatib, Rafiq Mourad, Hassan Derbani, *MEGBI Vaccine Pilot Plant – 2nd Project Report (Feb 2013 – Dec 2013),*

تصميم لمحطة انتاج لقاح HBsAg اولية

- Downstream processing elements for Hepatitis B DNA vaccine production process
- Process Chromatographic Purification Device
- Bioreactor Automation

[MEGBI-VPP 2014-15] Samir Mourad, *MEGBI Vaccine Pilot Plant – 3rd Project Report*

20.7 Other References

[Walsh 2007] Gary Walsh, "Pharmaceutical Biotechnology – Concepts and Applications", Wiley, Chichester/England, 2007

[Hass, Pörtner 2011] Volker C. Hass, Ralf Pörtner, „Praxis der Bioprozesstechnik – mit virtuellem Praktikum", 2. Auflage, Spektrum Akademischer Verlag, Heidelberg, 2011

[Mourad 2003] Samir Mourad, "Purification and characterization of a Glucose-I phosohatase from *Pantoea Agglomerans*", Karlsruhe, 2003, www.aecenar.com/publications

http://www.biopharminternational.com/biopharm/Downstream+Processing/Recombinant-Vaccine-Production-in-Yeast/ArticlesStandard/Article/detail/485189

[Patent Engerix B] Patent Engerix B (rHBsAg produced in S. cerevisiae)" SmithKline Beecham, 1998 (US), vaccination against hepatitis B

[Braun 2001] Fluoreszenz-optimierte Detektion heterologer Protein-Protein Interaktionen in *Saccharomyces cerevisiae* als Alternative zur Yeast Two Hybrid Methode DISSERTATION der Fakultät für Chemie und Pharmazie der Eberhard-Karls-Universität Tübingen zur Erlangung des Grades eines Doktors der Naturwissenschaften 2001

vorgelegt von Manuela Braun

Datum der mündlichen Prüfung 14. Mai 2001

Dekan Prof. Dr. H. Probst

1. Berichterstatter Prof. Dr. J. P. Ruppersberg

2. Berichterstatter Prof. Dr. D. Mecke

[Walsh 2007] Gary Walsh, "Pharmaceutical Biotechnology – Concepts and Applications", Wiley, Chichester/England, 2007

[AKTA Process Technical Manual]

Most Devices manufactor contacts from www.alibaba.com

[1]http://www.aecenar.com/publications

[2] http://www.aecenar.com/downloads/cat_view/7-megbi-institute

[3] http://www.aecenar.com/downloads/cat_view/3-meae-institute?start=10

[4]http://www.eio.com/p-1603-velleman-k8061-extended-usb-interface-board.aspx

[5]http://www.apogeekits.com/images/usb_io_interface.jpg

[6] www.pc-control.co.uk/relays.htm

[7] http://www.velleman.eu/products/view/?id=364910

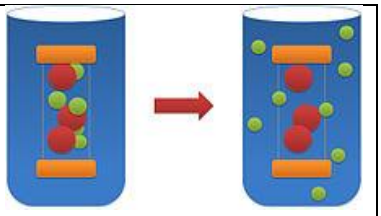
[8]http://www.tutorialspoint.com/python/

[9] https://www.draw.io/

SOFTWARES AND VIDEO DOCUMENTATION ARE FOUND ON THE DISC SUPPLIED WITH THIS DOCUMENT IN THE LAST PAGE

قموس المصطلحات (Dictionnary English - Arab)

Please see http://www.arab-ency.com/

dialysis	in biochemistry, **dialysis** is the process of separating molecules in solution by the difference in their rates of diffusionthrough a semipermeable membrane, such as dialysis tubing.	
cellular		خلوية
detergents	Reinigungsmittel	المنظّفات
dialysis		
protein expression		تعبير بروتيني
heterologous protein		بروتين لجين خارجي
thioredoxin		ذيوريدوكسين
plasmid		بلازميد
fusion protein		البروتين الانصهاري
Medium (Media)/ Culture Medium	Medium	مستنبت
purification		تنقية
incubation		احتضان
hydrophobic		هيدروفوبية
stimulated		محفز
Ammonium sulfate precipitation	**Ammonium sulfate precipitation** is a method used to purify proteins by altering their solubility. It is a specific case of a more general technique known as salting out.	ترقيد كبريتات الأمونيوم
recombinant		مؤتلف
glycoproteins		البروتينات السكّرية
Mannose		مانُّوز

immunogenic		
initialization		الاستهلال
sensors	Sensoren	اجهزة الاحساس

to research : Ti 15 rotor from Beckman